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(71) Applicant: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).			
(72) Inventors: WOLFEL, Thomas; Langenbeckstrasse 1, D-6500 Mainz (DE). VAN PEL, Aline; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). BRICHARD, Vincent; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE). BOON-FALLEUR, Thierry; Avenue Hippocrate 74, UCL 7459, B-1200 Brussels (BE).			
(74) Agent: HANSON, Norman, D.; Felfe & Lynch, 805 Third Avenue, New York, NY 10022 (US).			

(54) Title: ISOLATED, TYROSINASE DERIVED PEPTIDES AND USES THEREOF

(57) Abstract

The invention relates to the identification of complexes of human leukocyte antigen molecules and tyrosinase derived peptides on the surfaces of abnormal cells. The therapeutic and diagnostic ramifications of this observation are the subject of the invention.

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5 ISOLATED, TYROSINASE DERIVED PEPTIDES
AND USES THEREOF

RELATED APPLICATION

FIELD OF THE INVENTION

This invention relates to isolated peptides, derived from tyrosinase which are presented by HLA-A2 and HLA-B44 molecules and uses thereof. In addition, it relates to the ability to identify those individuals diagnosed with conditions characterized by cellular abnormalities whose abnormal cells present complexes of these peptides and HLA-A2 and HLA-B44, the presented peptides, and the ramifications thereof.

BACKGROUND AND PRIOR ART

25 The process by which the mammalian immune system
recognizes and reacts to foreign or alien materials is a
complex one. An important facet of the system is the T cell
response. This response requires that T cells recognize and
interact with complexes of cell surface molecules, referred to
30 as human leukocyte antigens ("HLA"), or major
histocompatibility complexes ("MHCs"), and peptides. The
peptides are derived from larger molecules which are processed
by the cells which also present the HLA/MHC molecule. See in
this regard Male et al., Advanced Immunology (J.P. Lipincott
35 Company, 1987), especially chapters 6-10. The interaction of
T cell and complexes of HLA/peptide is restricted, requiring
a T cell specific for a particular combination of an HLA
molecule and a peptide. If a specific T cell is not present,
there is no T cell response even if its partner complex is
40 present. Similarly, there is no response if the specific
complex is absent, but the T cell is present. This mechanism

5 is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Recently, much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, *Science* 257: 10 880 (1992); Fremont et al., *Science* 257: 919 (1992); Matsumura et al., *Science* 257: 927 (1992); Latron et al., *Science* 257: 964 (1992).

15 The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. 20 The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., *Immunogenetics* 35: 145 (1992); 25 van der Bruggen et al., *Science* 254: 1643 (1991), for further information on this family of genes.

30 In U.S. patent application Serial Number 938,334, the disclosure of which is incorporated by reference, nonapeptides are taught which bind to the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind one HLA molecule, but not others. 35 This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some 40 knowledge of the phenotype of the abnormal cells at issue.

The enzyme tyrosinase catalyzes the reaction converting

5 tyrosine to dehydroxyphenylalanine or "DOPA" and appears to be
expressed selectively in melanocytes (Muller et al., EMBOJ 7:
2715 (1988)). An early report of cDNA for the human enzyme is
found in Kwon, U.S. Patent No. 4,898,814. A later report by
Bouchard et al., J. Exp. Med. 169: 2029 (1989) presents a
10 slightly different sequence. A great deal of effort has gone
into identifying inhibitors for this enzyme, as it has been
implicated in pigmentation diseases. Some examples of this
literature include Jinbow, WO9116302; Mishima et al., U.S.
15 Patent No. 5,077,059, and Nazzaropor, U.S. Patent No.
4,818,768. The artisan will be familiar with other references
which teach similar materials.

U.S. Patent Application 08/081,673, filed June 23, 1993
and incorporated by reference, teaches that tyrosinase may be
treated in a manner similar to a foreign antigen or a TRAP
20 molecule - i.e., it was found that in certain cellular
abnormalities, such as melanoma, tyrosinase is processed and
a peptide derived therefrom forms a complex with HLA molecules
on certain abnormal cells. These complexes were found to be
25 recognized by cytolytic T cells ("CTLs"), which then lyse the
presenting cells. The ramifications of this surprising and
unexpected phenomenon were discussed. Additional peptides
have now been found which also act as tumor rejection antigens
30 presented by HLA-A2 molecules. These are described in Serial
No. 08/203,054, filed February 28, 1994 and incorporated by
reference.

It has now been found that additional peptides derived
from tyrosinase are tumor rejection antigens in that they are
presented by MHC molecule HLA-B44, and are lysed by cytolytic
T cells.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 describes, collectively, cell lysis studies. In particular:

10 Figure 1A shows lysis of cell line LB24-MEL;

Figure 1B shows lysis of cell line SK29-MEL;

Figure 1C shows lysis of cell line LB4.MEL;

Figure 1D shows lysis of cell line SK23.MEL;

Figure 1E shows lysis of cell line LE516.MEL;

15 Figure 1F shows lysis of cell line SK29-MEL.1.22 which has lost HLA-A2 expression;

Figure 1G shows lack of lysis of MZ2-MEL;

Figure 1H shows lysis studies on NK target K562;

Figure 1I shows lysis of the loss variant in Figure 1F after transfection with a gene for HLA-A2;

20 Figure 1J shows lysis of autologous EBV transformed B cells from patient SK29.

Figure 2 presents studies of TNF release of CTL IVSB.

Figure 3 depicts studies of TNF release of CTL 210/9.

25 Figure 4 depicts the recognition of the peptide YMNGTMSQV by cytolytic T cell clone CTL-IVSB but not cytolytic T cell clone CTL 2/9.

Figure 5 shows that the peptide YMNGTMSQV is not recognized by cytolytic T cell clone CTL 210/9.

30 Figure 6 shows the results obtained when TNF release assays were carried out on various cells, including those which present HLA-B44 on their surface.

Figure 7 shows, collectively, a series of chromium release assays using peptides described in this application on three different cell lines.

35 Figure 7A presents experiments where the peptide of SEQ ID NO: 4 was used.

Figure 7B shows results where the peptide of SEQ ID NO: 5 was used.

40 Figure 7C sets forth results obtained using SEQ ID NO: 2. In Figure 7, the symbol "O" is used for cell line T2, "■" for MZ2-MEL not presenting HLA-A2, and "●" for MZ2-MEL which has been transfected to present HLA-A2. Example 12

5 elaborates on these tests.

Figures 8A and 8B show work using a cell line which presents MHC molecule HLA-B44, and cytolytic T cell clone 22/31 ("CTL 22/31" hereafter). In figure 8A, the cell line ("Rosi EBV") was preincubated with monoclonal antibody W6/32, whereas in figure 8B, there was no preincubation.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

15 Melanoma cell lines SK 29-MEL (also referred to in the literature as SK MEL-29) and LB24-MEL, which have been available to researchers for many years, were used in the following experiments.

20 Samples containing mononuclear blood cells were taken from patients SK29 (AV) and LB2 (these patients were also the source of SK 29-MEL and LB24-MEL, respectively). The melanoma cell lines were contacted to the mononuclear blood cell containing samples. The mixtures were observed for lysis of the melanoma cell lines, this lysis indicating that cytolytic T cells ("CTLs") specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

25 The lysis assay employed was a chromium release assay following Herin et al., Int. J. Cancer 39:390-396 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown in vitro, and then resuspended at 10^7 cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200 μ Ci/ml of Na(⁵¹Cr)O₄. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These were then resuspended in DMEM supplemented with 10 mM Hepes and 10% FCS, after which 100 μ l aliquots containing 10^3 cells, were distributed into 96 well microplates. Samples of PBLs were added in 100 μ l of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and incubated for four hours at 37°C in a 5.5% of CO₂ atmosphere.

5 Plates were centrifuged again, and 100 ul aliquots of supernatant were collected and counted. Percentage of ^{51}Cr release was calculated as follows:

$$\% \text{ }^{51}\text{Cr} \text{ release} = \frac{(\text{ER}-\text{SR})}{(\text{MR}-\text{SR})} \times 100$$

10 where ER is observed, experimental ^{51}Cr release, SR is spontaneous release measured by incubating 10^3 labeled cells in 200 ul of medium alone, and MR is maximum release, obtained by adding 100 ul 0.3% Triton X-100 to target cells.

15 Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology.

20 The same method was used to test target K562 cells. When EBV-transformed B cells (EBV-B cells) were used, the only change was the replacement of DMEM medium by Hank's medium, supplemented with 5% FCS.

These experiments led to isolation of CTL clone "IVSB" from patient SK29 (AV) and CTL clone 210/9 from patient LB24.

25 Figure 1 presents the results of these assays, in panels A, B, G and I. Specifically, it will be seen that both CTLs lysed both melanoma cell lines, and that there was no lysis of the K562 and EBVB cell lines.

Example 2

30 The CTLs described were tested against other melanoma cell lines to determine whether their target was shared by other melanoma cell lines. Lysis as described in Example 1 was studied for lines LB4.MEL, SK23.MEL (also known as SK MEL-23), and LE516.MEL. Figure 1, panels C, D and E shows that the clones did lyse these lines.

35 The tested lines are known to be of type HLA-A2, and the results suggested that the CTLs are specific for a complex of peptide and HLA-A2. This suggestion was verified by testing a variant of SK 29-MEL which has lost HLA-A2 expression. Figure 1, panel F shows these results. Neither clone lysed the HLA-loss variant. When the variant was transfected with the HLA-A2 gene of SK29-MEL, however, and retested, lysis was observed. Thus, it can be concluded that the presenting

5 molecule is HLA-A2.

Example 3

10 Once the presenting HLA molecule was identified, studies were carried out to identify the molecule, referred to hereafter as the "tumor rejection antigen precursor" or "TRAP" molecule which was the source of the presented peptide.

15 To do this, total RNA was isolated from cell line SK29-MEL.1, which is a subclone of SK29-MEL. The RNA was isolated using an oligo-dT binding kit, following well recognized techniques. Once the total RNA was secured, it was transcribed into cDNA, again using standard methodologies. The cDNA was then ligated to EcoRI adaptors and cloned into the EcoRI site of plasmid pcDNA-I/Amp, in accordance with manufacturer's instructions. The recombinant plasmids were then electroporated into JM101 *E. coli* (electroporation 20 conditions: 1 pulse at 25 μ farads, 2500 V).

25 The transfected bacteria were selected with ampicillin (50 μ g/ml), and then divided into 700 pools of 200 clones each. Each pool represented about 100 different cDNAs, as analysis showed that about 50% of plasmids contained an insert. Each pool was amplified to saturation, and plasmid DNA was isolated via alkaline lysis, potassium acetate precipitation and phenol extraction, following Maniatis et al., in Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, N.Y., 1982). Cesium gradient centrifugation was not 30 used.

Example 4

35 The amplified plasmids were then transfected into eukaryotic cells. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were incubated overnight at 40 37°C, medium was removed and then replaced by 30 μ l/well of DMEM medium containing 10% Nu serum, 400 μ g/ml DEAE-dextran, 100 μ M chloroquine, 100 ng of plasmid pcDNA-I/Amp-A2 and 100 ng of DNA of a pool of the cDNA library described supra. Plasmid pcDNA-I/Amp-A2 contains the HLA-A2 gene from SK29-MEL.

5 Following four hours of incubation at 37°C, the medium was removed, and replaced by 50 µl of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200 µl of DMEM supplemented with 10% of FCS.

10 Following this change in medium, COS cells were incubated for 48 hours at 37°C. Medium was then discarded, and 2000 cells of either of the described CTL clones were added, in 100 µl of Iscove medium containing 10% pooled human serum. When clone 210/9 was used, the medium was supplemented with 25 U/ml of IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference.

15 Of 700 wells tested with IVSB, 696 showed between 0.6 and 4 pg of TNF per ml. The remaining four wells contained between 10 and 20 pg/ml of TNF. Homologous wells tested with CTL 210/9 showed similar, clearly higher values. Figures 2 and 3 present these data.

Example 5

20 Three of the four pools identified as high producers (numbers "123", "181" and "384") were selected for further experiments. Specifically, the bacteria were cloned, and 570 bacteria were tested from each pool. Plasmid DNA was extracted therefrom, transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of CTL 210/9 and CTL IVSB. A positive clone was found in pool 123 ("p123.B2"), and one was found in pool 384 ("p384.C6"). Convincing evidence that the transfected cells were recognized by CTLs was obtained by carrying out a comparative test of COS cells transfected with cDNA and the HLA-A2 gene, and COS cells transfected only with HLA-A2. TNF release in CTL supernatant was measured by testing it on WEHI cells. The optical density of the surviving WEHI cells was measured using MTT. Results are presented in Table 1:

Table 1

	CDNA (123.B2) + HLA-A2 DNA	no CDNA+ HLA-A2
10	Run 1 0.087	0.502
	Run 2 0.108	0.562

15 The values for WEHI OD's correspond to 24 pg/ml of TNF for CDNA and HLA-A2, versus 2.3 pg/ml for the control.

20 The plasmids from the positive clones were removed, and sequenced following art known techniques. A sequence search revealed that the plasmid insert was nearly identical to the 25 CDNA for human tyrosinase, as described by Bouchard et al., J. Exp. Med. 169: 2029 (1989), the disclosure of which is incorporated by reference. Thus, a normally occurring molecule (i.e., tyrosinase), may act as a tumor rejection antigen precursor and be processed to form a peptide tumor rejection antigen which is presented on the surface of a cell, in combination with HLA-A2, thereby stimulating lysis by CTL clones. The nucleic sequence of the identified molecule is presented as SEQ ID NO: 1.

25 Example 6

30 Prior work reported by Chomez et al., Immunogenetics 35: 241 (1992) has shown that small gene fragments which contain a sequence coding for an antigenic peptide resulted in expression of that peptide. This work, which is incorporated by reference in its entirety, suggested the cloning of small 35 portions of the human tyrosinase cDNA described supra and in SEQ ID NO: 1. Using the methodologies described in examples 1-5, various fragments of the cDNA were cotransfected with a gene for HLA-A2 in COS-7 cells, and TNF release assays were performed. These experiments led to identification of an 40 approximately 400 base pair fragment which, when used in cotransfection experiments, provoked TNF release from

10

5 cytolytic T cell clone CTL IVSB discussed supra, shown to be
specific for HLA-A2 presenting cells. The 400 base fragment
used corresponded to bases 711 to 1152 of SEQ ID NO: 1. The
amino acid sequence for which the fragment codes was deduced,
and this sequence was then compared to the information
provided by Hunt et al., Science 255: 1261 (1992), and Falk et
al., Nature 351: 290 (1991), the disclosures of which are both
incorporated by reference in their entirety. These references
discuss consensus sequences for HLA-A2 presented peptides.
Specifically, Hunt discusses nonapeptides, where either Leu or
10 Ile is always found at the second position, Leu being the
"dominant residue". The ninth residue is described as always
being a residue with an aliphatic hydrocarbon side chain. Val
is the dominant residue at this position. Hunt, discusses a
strong signal for Leu and an intermediate signal for Met at
15 the second position, one of Val, Leu, Ile or Thr at position
6, and Val or Leu at position 9, with Val being particularly
strong. On the basis of the comparison, nonapeptides were
synthesized and then tested to see if they could sensitize
20 HLA-A2 presenting cells. To do so, tyrosinase loss variant
cell lines SK29-MEL 1.218 and T202LB were used. Varying
concentrations of the tested peptides were added to the cell
lines, together with either of cytolytic T cell clone CTL IVSB
25 or cytolytic T cell clone CTL 2/9. Prior work, described
supra, had established that the former clone lysed tyrosinase
expressing cells which present HLA-A2, and that the latter did
30 not.

35 The tyrosinase loss variants were incubated for one hour in a solution containing ^{51}Cr , at 37°C, either with or without anti HLA-A2 antibody MA2.1, which was used to stabilize empty HLA-A2 molecules. In the tests, cells were washed four times, and then incubated with varying dilutions of the peptides, from 100 μM down to 0.01 μM . After 30 minutes, effector cells were added at an E/T ratio of 40/1 and four hours later, 100 λ of supernatant were collected and radioactivity counted.

40 Figure 4 shows the results obtained with nonapeptide

5 Tyr Met Asn Gly Thr Met Ser Gln Val.
(SEQ ID NO: 2).

10 This peptide, referred to hereafter as SEQ ID NO: 2, corresponds to residues 1129-1155 of the cDNA sequence for tyrosinase presented in SEQ ID NO: 1. Complexes of HLA-A2 and this peptide are recognized by CTL clone IVSB.

15 In a parallel experiment, it was shown that CTL clone CTL 210/9, derived from patient LB24, did not recognize the complexes of HLA-A2 and the peptide of SEQ ID NO: 2, although it did recognize complexes of HLA-A2 and a tyrosinase derived peptide. Thus, tyrosinase is processed to at least one additional peptide which, when presented by HLA-A2 molecules, is recognized by CTL clones.

20 Example 7

25 In a follow-up experiment, a second gene fragment which did not encode the peptide of SEQ ID NO: 2 was used. This fragment began at base 1 and ended at base 1101 of SEQ ID NO: 1 (i.e. the EcoRI-SphI fragment). Cytolytic T cell clone CTL 210/9, discussed supra, was tested against COS-7 cells transfected with this fragment in the manner described supra. CTL IVSB was also tested. These results, showed that LB24-CTL 210/9 recognized an antigen on the surface of HLA-A2 expressing cells transfected with this fragment, but CTL IVSB did not. Thus, a second tumor rejection antigen peptide is derived from tyrosinase.

30 Example 8

35 In order to further define the tumor rejection antigen recognized by LB24-CTL 210/9, the following experiments were carried out.

40 A second fragment, corresponding to bases 451-1158 of SEQ ID NO: 1 was transfected into COS cells together with a gene for HLA-A2, and TNF release assays were carried out. This sequence provoked TNF release from clone SK29-CTL IVSB (20 pg/ml), but not from LB24-CTL 210/9 (3.8 pg/ml). These results confirmed that the two CTL clones recognize different peptides, and that the peptide recognized by LB24-CTL 210/9 must be encoded by region 1-451.

5

Example 9

The tyrosinase derived peptide coded for by cDNA fragment 1-451 was analyzed for consensus sequences known to bind HLA-A2. The peptides corresponding to these consensus sequences were synthesized, and tested for their ability to sensitize HLA-A2 presenting cells. To do so, two tyrosinase negative melanoma cell lines were used (i.e., NA8-MEL, and MZ2-MEL 2.2 transfected with HLA-A2), and cell line T2, as described by Salter et al, Immunogenetics 21: 235-246 (1985)).

15 The cells were incubated with ^{51}Cr , and monoclonal antibody MA.2.1, which is specific for HLA-A2 for 50 minutes at 37°C, followed by washing (see Bodmer et al., Nature 342: 443-446 (1989), the disclosure of which is incorporated by reference in its entirety). Target cells were incubated with various concentrations of the peptides, and with either of LB 20 24-CTL clones 210/5 or 210/9. The percent of chromium release was measured after four hours of incubation.

The peptide Met Leu Leu Ala Val Leu Tyr Cys Leu Leu (SEQ ID NO: 3) was found to be active.

25 In further experiments summarized here, CTL-IVSB previously shown to recognize YMNGTMSQV, did not recognize the peptide of SEQ ID NO: 3.

The results are summarized in Tables 2-4 which follow:

Table 2

30

Peptide

	YMNGTMSQV (1120-1155)	MLLAVLYCLL (25-54)
SK29-CTL-IVSB	+	-
LB24-CTL-210/5	-	+
LB24-CTL-210/9	-	+

Table 3

3/93-Lysis of MZ2-2.2-A2 sensitized with tyrosinase peptides by LB24-CTL 210/5 and 210/9, and SK29-CTL IVSB

Effectors	Peptides	Dose	MZ2.2.2-A2 + anti-A2*	
LB24-CTL 210/5 (44:1)	MLLAVLYCLL (LAUS 17-5)	10μM	18	
			3	17
			1	16
	YMNGTMSQV (MAINZ)	30M	1	
		10	1	
		3	1	
LB24-CTL 210/9 (30:1)	MLLAVLYCLL (LAUS 17-5)	10μM	18	
			3	17
			1	15
	YMNGTMSQV (MAINZ)	30M	1	
		10	1	
		3	1	
SK29-CTL IVSB (40:1)	MLLAVLYCLL (LAUS 17-5)	10μM	1	
			3	1
			1	1
	YMNGTMSQV (MAINZ)	30μM	68	
		10	68	
		3	62	

* Target cells were incubated with Cr51 and mono-Ab MA2.1 (anti-HLA-A2) for 50 min, then washed 3 times. They were incubated with various concentrations of peptides for 30 min

CTL cells were added at the indicated (E:T) ratio.

The % specific Cr51 release was measured after 4h incubation

Table 4

8/93 : Test of tyrosinase peptides recognized by LB24-CTL 210/5 and 210/9 or SK29-CTL IVSB

(% Cr51 specific release)

Effectors	Peptides	Dose	NAB-MEL *	MZ2-2.2: A2	T2
LB24-CTL. 210/5 (41:1)	MLLAVLYCLL (LAUS 17-5)	10µM	30	31	36
		3	23	27	35
		1	17	20	26
		300nM	6	17	16
		100	2	8	5
		30	3	5	2
		0	0	0	0
LB24-CTL. 210/9 (26:1)	MLLAVLYCLL (LAUS 17-5)	10µM	14	19	21
		3	13	17	20
		1	9	14	13
		300nM	3	9	5
		100	1	1	1
		30	0	1	0
		0	0	1	0
SK29-CTL. IVSB (42:1)	YMNGTMSQV (MAINZ)	10µM	46	46	59
		3	38	44	52
		1	27	40	46
		300nM	14	22	34
		100	3	13	21
		30	1	9	10
		10	1	3	3
		3	0	3	4
		1	0	1	0
		0	0	4	0
spt. rel. max-spt %			339	259	198
			2694	1693	1206
			11	13	14

5 Example 10

10 Additional experiments were carried out using CTL clone 22/31. This clone had previously been shown to lyse subline MZ2-MEL.43 from autologous melanoma cell line MZ2-MEL, but did not lyse other sublines, such as MZ2-MEL 3.0 and MZ2-MEL 61.2, nor did it lyse autologous EBV transformed B cells, or killer cell line K562 (see Van den Eynde et al., Int. J. Cancer 44: 634-640 (1989)). The antigen presented by MZ2-MEL.43 is referred to as antigen C.

15 In prior work including that reported in the parent of this application, it was found that the tyrosinase gene encodes an antigen recognized by autologous CTLs on most HLA-A2 expressing melanomas. Expression of this gene in sublines of cell line MZ2-MEL was tested by PCR amplification. Clone MZ2-MEL.43 was found to be positive, whereas other MZ2-MEL clones, such as MZ2-MEL.3.0 were negative. Correlation of expression of the tyrosinase gene, and antigen MZ2-C, suggested that MZ2-C might be a tumor rejection antigen derived from tyrosinase, and presented by an HLA molecule expressed by MZ2-MEL. This cell line does not express HLA-A2, which would indicate that if a tyrosinase derived peptide were presented as a TRA, a second HLA molecule was implicated.

20 25 30 35 40 Studies were carried out to identify which HLA molecule presented antigen C to CTL 22/31. To determine this, cDNA clones of the HLA molecules known to be on the cell surface, i.e., HLA-A29, HLA-B37, HLA-B 44.02, and HLA-C clone 10, were isolated from an MZ2-MEL.43 cDNA library, and then cloned into expression vector pcDNA1/Amp. Recipient COS 7 cells were then transfected with one of these constructs or a construct containing HLA-A1, plus cDNA coding for tyrosinase (SEQ ID NO: 1). The contransfection followed the method set forth above. One day later CTL 22/31 was added, and 24 hours later, TNF release was measured by testing cytotoxicity on WEHI-164-13, following Traversari et al, supra. Figure 6 shows that TNF was released by CTL 22/31 only in the presence of cells transfected with both HLA-B44 and tyrosinase. The conclusion to be drawn from this is that HLA-B44 presents a tyrosinase

5 derived tumor rejection antigen.

Example 11

10 The experiments described supra showed, inter alia, that the decamer MLLAVLYCLL effectively induced lysis of HLA-A2 presenting cells. It is fairly well accepted that MHC molecules present nonapeptides. To that end, experiments were carried out wherein two nonamers were tested, which were based upon the decapeptide which did give positive results. Specifically, either the first or tenth amino acid was omitted to create two peptides, i.e.:

15 Met Leu Leu Ala Val Leu Tyr Cys Leu

(SEQ ID NO: 4)

Leu Leu Ala Val Leu Tyr Cys Leu Leu

(SEQ ID NO: 5).

20 These peptides were tested in the same way the decapeptide was tested, as set forth in the prior examples at concentrations ranging from 10 μ M to 1 nM. Three presenting cells were used. As summarized in Table 5, which follows, "T2" is a mutant human cell line, "CEMX721.174T2" as described by Salter, Immunogenetics 21: 235(1985). This line presents HLA-A2. "G2.2" is a variant of the cell line MZ2-MEL. The variant has been transfected with a gene coding for HLA-A2. The abbreviation "G2.2.5" stands for a variant which does not express HLA-A2. All cells were incubated with monoclonal antibody MA2.1 prior to contact with the cytolytic T cell clone. This procedure stabilizes so-called "empty" MHC molecules, although the mechanism by which this occurs is not well understood and effector CTLs 210/5 and 210/9 were both used. The results are set forth in Table 5, which follows. They show that at a concentration of 10 μ M, the nonamer of SEQ ID NO: 4 was twice as effective when used with CTL clone 210/5, and four times as effective with clone 210/9 whereas the nonamer of SEQ ID NO: 5 was ineffective at inducing lysis.

Example 12

40 In further experiments, chromium release assays were carried out using the peptides of SEQ ID NOS: 4 and 5, as well as SEQ ID NO: 2. The target cells were allogeneic melanoma

5 cells, i.e., MZ2-MEL, previously transfected with HLA-A2, and
cell line T2, which presents HLA-A2, but has an antigen
processing defect which results in an increased capacity to
present exogenous peptides (Cerundolo et al., Nature 345: 449
10 (1990)). All cells were pretreated with monoclonal antibody
MA2.1 for fifty minutes. The cells were incubated with the
peptide of choice, for 30 minutes, at various concentrations.
Then, one of CTL clones 210/9 and ISVB was added in an
effector: target ratio of 60. Chromium release was measured
15 after four hours, in the manner described supra.

15 The results are presented in figure 7, i.e., figures 7A-
7C. The peptide of SEQ ID NO: 4 sensitized cells to CTL
210/9, while SEQ ID NO: 5 did not. SEQ ID NO: 6 sensitized
cells to CTL IVSB, as already noted in previous examples.

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TABLE 5

1	2	3	4	5	6	7	8	9	10	11	12	13
Efficacité	Peptide	Peptide	Doce	T2 +e-A2	G22.5+ e-A2	G22.5+ e-A2	Efficacité	Peptide	Doce	T2 +e-A2	G22.5+ e-A2	G22.5+ e-A2
1 DAGI 210/5	LLAVYCL	10 μ M	(LAUS 17-5)	3	50	32	3	SK20 IVSB	LLAVYCL	10 μ M	3	3
2 50:1				1	45	32	5	(LAUS 17-5)		0	2	7
3				1	39	28	3			2	3	4
4				1	33	18	4			1	1	6
5				1	10	4	4			2	2	7
6				1	5	5	5			1	2	7
7				1	5	5	5			1	4	7
8				1	5	5	5			0	3	7
9				1	4	4	4			0	4	7
10				1	3	2	3			2	4	7
11				1	2	1	1			2	2	7
12				1	1	1	1			1	1	7
13				1	1	1	1			1	1	1
14				1	1	1	1			1	1	1
15				1	1	1	1			1	1	1
16				1	1	1	1			1	1	1
17				1	1	1	1			1	1	1
18				1	1	1	1			1	1	1
19				1	1	1	1			1	1	1
20				1	1	1	1			1	1	1
21				1	1	1	1			1	1	1
22				1	1	1	1			1	1	1
23				1	1	1	1			1	1	1
24				1	1	1	1			1	1	1
25				1	1	1	1			1	1	1
26				1	1	1	1			2	2	6
27				1	1	1	1			1	1	5
28				1	1	1	1			1	1	4
29				1	1	1	1			1	1	10
30				1	1	1	1			1	1	7

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TABLE 5 - Cont'd

Effecteur	Pepide	Dose	T2 α -A2			G2.2 α -A2			G2.2.5 α -A2			Effecteur			Pepide	Dose	T2 α -A2			G2.2 α -A2			G2.2.5 α -A2																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	10

TABLE 5 - Cont'd

Effecteur	Peptide	Dose	12 +e-A2	G2.2 +e-A2	G2.2.5+ e-A2	Effecteur	Peptide	Dose	12 +e-A2	G 2.2 +e-A2	G 2.2.5 +e-A2
61	61	10	82	42	6	61	61	1	3	7	6
61	61	3	87	34	6	61	61	1	3	6	6
61	61	1	77	30	7	61	61	1	4	6	6
300pg			70	25	7	300nM		5	3	3	3
100			73	27	6	100		2	1	1	1
30			41	22	6	30		1	1	1	1
SHAMYCL 10µM (Lane 16-19)			1	1	1	YANGTSEGY 10µM (MANZ)		6	3	6	6
3			1	1	1	3		5	4	7	6
1			1	1	1	1		2	5	5	6
300nM			1	1	1	300nM		2	2	4	4
100			2	2	2	100		2	2	7	7
30			1	1	1	30		2	3	3	3
71	71	71	72	72	72	10	10	2	2	7	7
71	71	71	73	73	73	3	3	3	3	6	6
74	74	74	75	75	75	1	1	1	1	1	1
74	74	74	76	76	76	6	6	6	6	6	6
74	74	74	77	77	77	0	0	0	0	0	0
74	74	74	78	78	78	0	0	0	0	0	0
74	74	74	79	79	79	0	0	0	0	0	0
74	74	74	80	80	80	0	0	0	0	0	0
74	74	74	81	81	81	0	0	0	0	0	0
74	74	74	82	82	82	0	0	0	0	0	0
74	74	74	83	83	83	0	0	0	0	0	0
74	74	74	84	84	84	0	0	0	0	0	0
74	74	74	85	85	85	0	0	0	0	0	0
74	74	74	86	86	86	0	0	0	0	0	0
74	74	74	87	87	87	0	0	0	0	0	0
74	74	74	88	88	88	0	0	0	0	0	0
74	74	74	89	89	89	0	0	0	0	0	0
74	74	74	90	90	90	0	0	0	0	0	0
74	74	74	91	91	91	0	0	0	0	0	0
74	74	74	92	92	92	0	0	0	0	0	0
74	74	74	93	93	93	0	0	0	0	0	0
74	74	74	94	94	94	0	0	0	0	0	0
74	74	74	95	95	95	0	0	0	0	0	0
74	74	74	96	96	96	0	0	0	0	0	0
74	74	74	97	97	97	0	0	0	0	0	0
74	74	74	98	98	98	0	0	0	0	0	0
74	74	74	99	99	99	0	0	0	0	0	0
74	74	74	100	100	100	0	0	0	0	0	0
74	74	74	101	101	101	0	0	0	0	0	0
74	74	74	102	102	102	0	0	0	0	0	0
74	74	74	103	103	103	0	0	0	0	0	0
74	74	74	104	104	104	0	0	0	0	0	0
74	74	74	105	105	105	0	0	0	0	0	0
74	74	74	106	106	106	0	0	0	0	0	0
74	74	74	107	107	107	0	0	0	0	0	0
74	74	74	108	108	108	0	0	0	0	0	0
74	74	74	109	109	109	0	0	0	0	0	0
74	74	74	110	110	110	0	0	0	0	0	0
74	74	74	111	111	111	0	0	0	0	0	0
74	74	74	112	112	112	0	0	0	0	0	0
74	74	74	113	113	113	0	0	0	0	0	0
74	74	74	114	114	114	0	0	0	0	0	0
74	74	74	115	115	115	0	0	0	0	0	0
74	74	74	116	116	116	0	0	0	0	0	0
74	74	74	117	117	117	0	0	0	0	0	0
74	74	74	118	118	118	0	0	0	0	0	0
74	74	74	119	119	119	0	0	0	0	0	0
74	74	74	120	120	120	0	0	0	0	0	0
74	74	74	121	121	121	0	0	0	0	0	0
74	74	74	122	122	122	0	0	0	0	0	0
74	74	74	123	123	123	0	0	0	0	0	0
74	74	74	124	124	124	0	0	0	0	0	0
74	74	74	125	125	125	0	0	0	0	0	0
74	74	74	126	126	126	0	0	0	0	0	0
74	74	74	127	127	127	0	0	0	0	0	0
74	74	74	128	128	128	0	0	0	0	0	0
74	74	74	129	129	129	0	0	0	0	0	0
74	74	74	130	130	130	0	0	0	0	0	0
74	74	74	131	131	131	0	0	0	0	0	0
74	74	74	132	132	132	0	0	0	0	0	0
74	74	74	133	133	133	0	0	0	0	0	0
74	74	74	134	134	134	0	0	0	0	0	0
74	74	74	135	135	135	0	0	0	0	0	0
74	74	74	136	136	136	0	0	0	0	0	0
74	74	74	137	137	137	0	0	0	0	0	0
74	74	74	138	138	138	0	0	0	0	0	0
74	74	74	139	139	139	0	0	0	0	0	0
74	74	74	140	140	140	0	0	0	0	0	0
74	74	74	141	141	141	0	0	0	0	0	0
74	74	74	142	142	142	0	0	0	0	0	0
74	74	74	143	143	143	0	0	0	0	0	0
74	74	74	144	144	144	0	0	0	0	0	0
74	74	74	145	145	145	0	0	0	0	0	0
74	74	74	146	146	146	0	0	0	0	0	0
74	74	74	147	147	147	0	0	0	0	0	0
74	74	74	148	148	148	0	0	0	0	0	0
74	74	74	149	149	149	0	0	0	0	0	0
74	74	74	150	150	150	0	0	0	0	0	0
74	74	74	151	151	151	0	0	0	0	0	0
74	74	74	152	152	152	0	0	0	0	0	0
74	74	74	153	153	153	0	0	0	0	0	0
74	74	74	154	154	154	0	0	0	0	0	0
74	74	74	155	155	155	0	0	0	0	0	0
74	74	74	156	156	156	0	0	0	0	0	0
74	74	74	157	157	157	0	0	0	0	0	0
74	74	74	158	158	158	0	0	0	0	0	0
74	74	74	159	159	159	0	0	0	0	0	0
74	74	74	160	160	160	0	0	0	0	0	0
74	74	74	161	161	161	0	0	0	0	0	0
74	74	74	162	162	162	0	0	0	0	0	0
74	74	74	163	163	163	0	0	0	0	0	0
74	74	74	164	164	164	0	0	0	0	0	0
74	74	74	165	165	165	0	0	0	0	0	0
74	74	74	166	166	166	0	0	0	0	0	0
74	74	74	167	167	167	0	0	0	0	0	0
74	74	74	168	168	168	0	0	0	0	0	0
74	74	74	169	169	169	0	0	0	0	0	0
74	74	74	170	170	170	0	0	0	0	0	0
74	74	74	171	171	171	0	0	0	0	0	0
74	74	74	172	172	172	0	0	0	0	0	0
74	74	74	173	173	173	0	0	0	0	0	0
74	74	74	174	174	174	0	0	0	0	0	0
74	74	74	175	175	175	0	0	0	0	0	0
74	74	74	176	176	176	0	0	0	0	0	0
74	74	74	177	177	177	0	0	0	0	0	0
74	74	74	178	178	178	0	0	0	0	0	0
74	74	74	179	179	179	0	0	0	0	0	0
74	74	74	180	180	180	0	0	0	0	0	0
74	74	74	181	181	181	0	0	0	0	0	0
74	74	74	182	182	182	0	0	0	0	0	0
74	74	74	183	183	183	0	0	0	0	0	0
74	74	74	184	184	184	0	0	0	0	0	0
74	74	74	185	185	185	0	0	0	0	0	0
74	74	74	186	186	186	0	0	0	0	0	0
74	74	74	187	187	187	0	0	0	0	0	0
74	74	74	188	188	188	0	0	0	0	0	0
74	74	74	189	189	189	0	0	0	0	0	0
74	74	74	190	190	190	0	0	0	0	0	0
74	74	74	191	191	191	0	0	0	0	0	0
74	74	74	192	192	192	0	0	0	0	0	0
74	74	74	193	193	193	0	0	0	0	0	0
74	74	74	194	194	194	0	0	0	0	0	0
74	74	74	195	195	195	0	0	0	0	0	0
74	74	74	196	196	196	0	0	0	0	0	0
74	74	74	197	197	197	0	0	0	0	0	0
74	74	74	198	198	198	0	0	0	0	0	0
74	74	74	199	199	199	0	0	0	0	0	0
74	74	74	200	200	200	0	0	0	0	0	0
74	74	74	201	201	201	0	0	0	0	0	0
74	74	74	202	202	202	0	0	0	0	0	0

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5 Example 13

Work which followed up on the experiments set forth in example 10 was then carried out, in an effort to define the antigenic peptide presented by HLA-B44. To do so, cDNA sequences corresponding to fragments of the tyrosinase cDNA sequence were cotransfected, together with a gene coding for HLA-B44, into COS-7 cells. The protocol is essentially that described in example 6, *supra*. The cytolytic T cell clone 22/31, discussed *supra*, was used. TNF release was determined. Two fragments, i.e., base fragments 1-611, and 427-1134 induced TNF release. This suggested that the presented peptide was in the overlapping region. As a result of this observation, shorter fragments were tested. Fragments beginning at positions 385, 442, 514 and 574 were able to induce TNF release, while fragments starting at positions 579 and 585 were not. These observations, in turn, suggested the synthesis, following standard methodologies, of a 13 amino acid peptide beginning at position 574.

20 This peptide was then used in experiments to determine whether it induced lysis by CTL 22/31. Table 6, which follows, shows that the 13-mer rendered two EBV transfected cell lines which express HLA-B44 sensitive to lysis.

Table 6

10F94-tyros 13-mer sur EBV-I		1	2	3	4
	Effector	Dose pept 13 A.A.	Ros1-EBV	MZ2-EBV	
10	1	MZ2-CTL-22/31 SEWRDIDFAHEA			
	2				
	3	60:1	80µM	83	71
	4		10	85	72
15	5		3	77	66
	6		1	79	63
	7		300nM	80	33
	8		100	44	17
	9		30	21	4
20	10		10	9	5
	11		3	10	6
	12		0	10	6
	13				
	14				
25	15	splrel.		393	472
	16	max.rel.		1898	1792
	17	%		23	26

30

As a follow up, even shorter peptides were tested. A decamer corresponding to nucleotide bases 574-604, i.e.

Ser Glu Ile Trp Arg Asp Ile Asp Phe Ala
(SEQ ID NO: 7)

35

did provoke lysis, as did peptide:

Ser Glu Ile Trp Arg Asp Ile Asp Phe
(SEQ ID NO: 8)

The nonamer:

Glu Ile Trp Arg Asp Ile Asp Phe Ala
(SEQ ID NO: 9)

40

in contrast, was not recognized. Table 7, which follows,

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5 summarizes these results, which are also depicted in figure 8.

The only other peptide reported to be bound by HLA-B44 is

Glu Glu Asn Leu Leu Asp Phe Val Arg Phe

(SEQ ID NO: 10)

10 as reported by Burrows et al., J. Virol 64: 3974 (1990). The data described supra suggest that Glu at second position and Phe in ninth position may represent anchor residues for HLA-B44.

15 Table 7

10M94-pept tyros on B44		1	2	3	4
	Effector	Dose	+W SEIWRDIDFA	+W SEMWRDIDF	
20	1 MZ2-CTL-22/31	1μM	91	93	
	2	300nM	78	81	
25	3 45:1	100	43	73	
	4	30	17	37	
	5	10	4	12	
	6	3	3	4	
	7	1	2	4	
30	8	0.3	1	1	

TABLE 7 - Cont'd

5 +W EIWRDIDFA	6 -W SEIWRDIDFA	7 -W SEIWRDIDF	8 -W EIWRDIDFA
7	98	99	11
4	77	97	6
2	45	64	8
0	15	21	6
1	5	8	4
1	0	7	2
		3	
		2	

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5 The foregoing experiments demonstrate that tyrosinase is processed as a tumor rejection antigen precursor, leading to formation of complexes of the resulting tumor rejection antigens with a molecule on at least some abnormal cells, for example, melanoma cells with HLA-A2 or HLA-B44 phenotype. The
10 complex can be recognized by CTLs, and the presenting cell lysed. This observation has therapeutic and diagnostic ramifications which are features of the invention. With respect to therapies, the observation that CTLs which are specific for abnormal cells presenting the aforementioned
15 complexes are produced, suggests various therapeutic approaches. One such approach is the administration of CTLs specific to the complex to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CTLs in vitro. Specifically, a sample of
20 cells, such as blood cells, are contacted to a cell presenting the complex and are capable of provoking a specific CTL to proliferate. The target cell can be a transfectant, such as a COS cell of the type described supra. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation.
25 So as to enable the artisan to produce these CTLs, vectors containing the genes of interest, i.e., pcDNA-1/Ampl (HLA-A2), and p123.B2 (human tyrosinase), have been deposited in accordance with the Budapest Treaty at the Institut Pasteur,
30 under Accession Numbers I1275 and I1276, respectively. COS cells, such as those used herein are widely available, as are other suitable host cells.

35 To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Reddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the

5 particular complex. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

10 The foregoing therapy assumes that at least some of the subject's abnormal cells present one or more of the HLA/tyrosinase derived peptide complexes. This can be determined very easily. For example CTLs are identified using the transfectants discussed supra, and once isolated, can be used with a sample of a subject's abnormal cells to determine lysis in vitro. If lysis is observed, then the use of specific CTLs in such a therapy may alleviate the condition 15 associated with the abnormal cells. A less involved methodology examines the abnormal cells for their HLA phenotype, using standard assays, and determines expression of tyrosinase via amplification using, e.g., PCR. The fact that a plurality of different HLA molecules present TRAs derived 20 from tyrosinase increases the number of individuals who are suitable subjects for the therapies discussed herein.

25 Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon supra. The cells used in this approach may be those that normally express the complex, such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the 30 complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114 (January, 1991) exemplifies this approach, showing the use of transfected cells expressing HPVE7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. 35 Viral or bacterial vectors are especially preferred. In these systems, the gene of interest is carried by, e.g., a Vaccinia virus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining 40 tyrosinase itself with an adjuvant to facilitate incorporation

5 into HLA-A2 presenting cells. The enzyme is then processed to yield the peptid partner of the HLA molecule.

10 The foregoing discussion refers to "abnormal cells" and "cellular abnormalities". These terms are employed in their broadest interpretation, and refer to any situation where the cells in question exhibit at least one property which indicates that they differ from normal cells of their specific type. Examples of abnormal properties include morphological and biochemical changes, e.g. Cellular abnormalities include tumors, such as melanoma, autoimmune disorders, and so forth.

15 The invention also provides a method for identifying precursors to CTL targets. These precursors are referred to as tumor rejection antigens when the target cells are tumors, but it must be pointed out that when the cell characterized by abnormality is not a tumor, it would be somewhat misleading to refer to the molecule as a tumor rejection antigen. Essentially, the method involves identifying a cell which is the target of a cytolytic T cell of the type discussed supra. Once such a cell is identified, total RNA is converted to a cDNA library, which is then transfected into a cell sample capable of presenting an antigen which forms a complex with a relevant HLA molecule. The transfectants are contacted with the CTL discussed supra, and again, targeting by the CTL is observed (lysis and/or TNF production). These transfectants which are lysed are then treated to have the cDNA removed and sequenced, and in this manner a precursor for an abnormal condition, such as a tumor rejection antigen precursor, can be identified.

30 Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

35 The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

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1) GENERAL INFORMATION:

(i) APPLICANTS: Wölfel, Thomas; Van Pel, Aline; Brichard, Vincent; Boon-Falleur, Thierry

(ii) TITLE OF INVENTION: ISOLATED, TYROSINASE DERIVED PEPTIDES AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Felfe & Lynch
(B) STREET: 805 Third Avenue
(C) CITY: New York City
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10022

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 5.25 inch, 360 kb storage
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: PC-DOS
(D) SOFTWARE: Wordperfect

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(C) CLASSIFICATION: 514

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29

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(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Hanson, Norman D.
- (B) REGISTRATION NUMBER: 30,946
- (C) REFERENCE/DOCKET NUMBER: LUD 5360.1

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (212) 688-9200
- (B) TELEFAX: (212) 838-3884

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30

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1894 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGA AGA ATG CTC CTG GCT GTT TTG TAC TGC CTG CTG TGG AGT TTC CAG	48
Gly Arg Met Leu Leu Ala Val Leu Tyr Cys Leu Leu Trp Ser Phe Gln	
15 -10 -5	
ACC TCC GCT GGC CAT TTC CCT AGA GCC TGT GTC TCC TCT AAG AAC CTG	96
Thr Ser Ala Gly His Phe Pro Arg Ala Cys Val Ser Ser Lys Asn Leu	
1 5 10	
ATG GAG AAG GAA TGC TGT CCA CCG TGG AGC GGG GAC AGG AGT CCC TGT	144
Met Gly Lys Glu Cys Cys Pro Pro Trp Ser Gly Asp Arg Ser Pro Cys	
15 20 25 30	
GGC CAG CTT TCA GGC AGA GGT TCC TGT CAG AAT ATC CTT CTG TCC AAT	192
Gly Gln Leu Ser Gly Arg Gly Ser Cys Gln Asn Ile Leu Leu Ser Asn	
35 40 45	
GCA CCA CTT GGG CCT CAA TTT CCC TTC ACA GGG GTG GAT GAC CGG GAG	240
Ala Pro Leu Gly Pro Gln Phe Pro Phe Thr Gly Val Asp Asp Arg Glu	
50 55 60	
TCG TGG CCT TCC GTC TTT TAT AAT AGG ACC TGC CAG TGC TCT GGC AAC	288
Ser Trp Pro Ser Val Phe Tyr Asn Arg Thr Cys Gln Cys Ser Gly Asn	
65 70 75	
TTC ATG GGA TTC AAC TGT GGA AAC TGC AAG TTT GGC TTT TGG GGA CCA	336
Phe Met Gly Phe Asn Cys Gly Asn Cys Lys Phe Gly Phe Trp Gly Pro	
80 85 90	
AAC TGC ACA GAG AGA CGA CTC TTG GTG AGA AGA AAC ATC TTC GAT TTG	384
Asn Cys Thr Glu Arg Arg Leu Leu Val Arg Arg Asn Ile Phe Asp Leu	
95 100 105 110	
AGT GCC CCA GAG AAG GAC AAA TTT TGT GCC TAC CTC ACT TTA GCA AAG	432
Ser Ala Pro Glu Lys Asp Lys Phe Phe Ala Tyr Leu Thr Leu Ala Lys	
115 120 125	

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CAT ACC ATC AGC TCA GAC TAT GTC ATC CCC ATA GGG ACC TAT GGC CAA	480
His Thr Ile Ser Ser Asp Tyr Val Ile Pro Ile Gly Thr Tyr Gly Gln	
130 135 140	
ATG AAA AAT GGA TCA ACA CCC ATG TTT AAC GAC ATC AAT ATT TAT GAC	528
Met Lys Asn Gly Ser Thr Pro Met Phe Asn Asp Ile Asn Ile Tyr Asp	
145 150 155	
CTC TTT GTC TGG ATG CAT TAT TAT GTG TCA ATG GAT GCA CTG CTT GGG	576
Leu Phe Val Trp Ile His Tyr Tyr Val Ser Met Asp Ala Leu Leu Gly	
160 165 170	
GGA TCT GAA ATC TGG AGA GAC ATT GAT TTT GCC CAT GAA GCA CCA GCT	624
Gly Tyr Glu Ile Trp Arg Asp Ile Asp Phe Ala His Glu Ala Pro Ala	
175 180 185 190	
TTT CTG CCT TGG CAT AGA CTC TTC TTG TTG CGG TGG GAA CAA GAA ATC	672
Phe Leu Pro Trp His Arg Leu Phe Leu Leu Arg Trp Glu Gln Gly Ile	
195 200 205	
CAG AAG CTG ACA GGA GAT GAA AAC TTC ACT ATT CCA TAT TGG GAC TGG	720
Gln Lys Leu Thr Gly Asp Gly Asn Phe Thr Ile Pro Tyr Trp Asp Trp	
210 215 220	
CGG GAT GCA GAA AAG TGT GAC ATT TGC ACA GAT GAG TAC ATG GGA GGT	768
Arg Asp Ala Glu Lys Cys Asp Ile Cys Thr Asp Gly Tyr Met Gly Gly	
225 230 235	
CAG CAC CCC ACA AAT CCT AAC TTA CTC AGC CCA GCA TCA TTC TCC	816
Gln His Pro Thr Asn Pro Asn Leu Leu Ser Pro Ala Ser Phe Phe Ser	
240 245 250	
TCT TGG CAG ATT GTC TGT AGC CGA TTG GAG GAG TAC AAC AGC CAT CAG	864
Ser Trp Gln Ile Val Cys Ser Arg Leu Glu Glu Tyr Asn Ser His Gln	
255 260 265 270	
TCT TTA TGC AAT GGA ACG CCC GAG GGA CCT TTA CGG CGT AAT CCT GGA	912
Ser Leu Cys Asn Gly Thr Pro Glu Gly Pro Leu Arg Arg Asn Pro Gly	
275 280 285	
AAC CAT GAC AAA TCC AGA ACC CCA AGG CTC CCC TCT TCA GCT GAT GTA	960
Asn His Asp Lys Ser Arg Thr Pro Arg Leu Pro Ser Ser Ala Asp Val	
290 295 300	
GAA TTT TGC CTG AGT TTG ACC CAA TAT GAA TCT GGT TCC ATG GAT AAA	1008
Glu Phe Cys Leu Ser Leu Thr Gln Tyr Glu Ser Gly Ser Met Asp Lys	
305 310 315	
GCT GCC AAT TTC AGC TTT AGA AAT ACA CTG GAA GGA TTT GCT AGT CCA	1056

Ala	Ala	Asn	Phe	Ser	Phe	Arg	Asn	Thr	Leu	Glu	Gly	Phe	Ala	Ser	Pro	
320					325					330						
CTT	ACT	GGG	ATA	GCG	GAT	GCC	TCT	CAA	AGC	AGC	ATG	CAC	AAT	GCC	TTG	1104
Leu	Thr	Gly	Ile	Ala	Asp	Ala	Ser	Gln	Ser	Ser	Met	His	Asn	Ala	Leu	
335					340				345			350				
CAC	ATC	TAT	ATG	AAT	GGA	ACA	ATG	TCC	CAG	GTA	CAG	GGA	TCT	GCC	AAC	1152
His	Ile	Tyr	Met	Asn	Gly	Thr	Met	Ser	Gln	Met	Gln	Gly	Ser	Ala	Asn	
					355				360			365				
GAT	CCT	ATC	TTC	CTT	CTT	CAC	CAT	GCA	TTT	GTT	GAC	AGT	ATT	TTT	GAG	1200
Asp	Pro	Ile	Phe	Leu	Leu	His	His	Ala	Phe	Val	Asp	Ser	Ile	Phe	Glu	
					370				375			380				
CAG	TGG	CTC	CAA	AGG	CAC	CGT	CCT	CAA	GAA	GTT	TAT	CCA	GAA	GCC		1248
Gln	Trp	Leu	Arg	Arg	His	Arg	Pro	Leu	Gln	Glu	Val	Tyr	Pro	Glu	Ala	
					385				390			395				
AAT	GCA	CCC	ATT	GGA	CAT	AAC	CGG	GAA	TCC	TAC	ATG	GTT	CCT	TTT	ATA	1296
Asn	Ala	Pro	Ile	Gly	His	Asn	Arg	Glu	Ser	Tyr	Met	Val	Pro	Phe	Ile	
					400				405			410				
CCA	CTG	TAC	AGA	AAT	GGT	GAT	TTC	TTT	ATT	TCA	TCC	AAA	GAT	CTG	GGC	1344
Pro	Leu	Tyr	Arg	Asn	Gly	Asp	Phe	Phe	Ile	Ser	Ser	Lys	Asp	Leu	Gly	
					415				420			425			430	
TAT	GAC	TAT	AGC	TAT	CTA	CAA	GAT	TCA	GAC	CCA	GAC	TCT	TTT	CAA	GAC	1392
Tyr	Asp	Tyr	Ser	Tyr	Leu	Gln	Asp	Ser	Asp	Pro	Asp	Ser	Phe	Gln	Asp	
					435				440			445				
TAC	ATT	AAG	TCC	TAT	TTG	GAA	CAA	GCG	AGT	CGG	ATC	TGG	TCA	TGG	CTC	1440
Tyr	Ile	Lys	Ser	Tyr	Leu	Gly	Gln	Ala	Ser	Arg	Ile	Trp	Ser	Trp	Leu	
					450				455			460				
CTT	GGG	GCG	GCG	ATG	GTA	GGG	GCC	GTC	CTC	ACT	GCC	CTG	CTG	GCA	GGG	1488
Leu	Gly	Ala	Ala	Met	Val	Gly	Ala	Val	Leu	Thr	Ala	Leu	Leu	Ala	Gly	
					465				470			475				
CTT	GTG	AGC	TTG	CTG	TGT	CGT	CAC	AAG	AGA	AAG	CAG	CTT	CCT	GAA	GAA	1536
Leu	Val	Ser	Leu	Leu	Cys	Arg	His	Lys	Arg	Lys	Gln	Leu	Pro	Glu	Glu	
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AAG	CAG	CCA	CTC	CTC	ATG	GAG	AAA	GAG	GAT	TAC	CAC	AGC	TTG	TAT	CAG	1584
Lys	Gln	Pro	Leu	Leu	Met	Glu	Lys	Glu	Asp	Tyr	His	Ser	Leu	Tyr	Gln	
					495				500			505			510	
AGC	CAT	TTA														1593

Ser His Leu

513

TAAAAGGCTT AGGCAATAGA GTAGGGCCAA AAAGCCTGAC CTCACTCTAA CTCAAAGTAA 1653
TGTCCAGGTT CCCAGAGAAT ATCTGCTGGT ATTTTCTGT AAAGACCATT TGCAAAATTG 1713
TAACCTAATA CAAAGTGTAG CCTTCTTCCA ACTCAGGTAG AACACACCTG TCTTTGTCTT 1773
GCTGTTTCA CTCAGCCCTT TTAACATTTT CCCCTAAGCC CATATGTCTA AGGAAAGGAT 1833
GCTATTGGT AATGAGGAAC TGTTATTTGT ATGTGAATTA AAGTGCTCTT ATTTAAAAA 1893
A 1894

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acid residues
- (B) TYPE: amino acid
- (D) TOPOLOGY: single

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Tyr Met Asn Gly Thr Met Ser Gln Val

5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acid residues
- (B) TYPE: amino acid
- (D) TOPOLOGY: single

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Leu Leu Ala Val Leu Tyr Cys Leu Leu

5

10

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Leu Leu Ala Val Leu Tyr Cys Leu

5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Leu Leu Ala Val Leu Tyr Cys Leu Leu

5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ser Glu Ile Trp Arg Asp Ile Asp Phe Ala His Glu Ala

5

10

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35

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acid residues
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ser Glu Ile Trp Arg Asp Ile Asp Phe Ala

5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acid residues
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ser Glu Ile Trp Arg Asp Ile Asp Phe

5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acid residues
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Glu Ile Trp Arg Asp Ile Asp Phe Ala

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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Glu Glu Asn Leu Leu Asp Phe Val Arg Phe

5

10

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We claim:

1. Method for identifying a candidate for treatment with a therapeutic agent specific for complexes of an MHC molecule and a tyrosinase derived peptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 7, and SEQ ID NO: 8 comprising:

(i) contacting an abnormal cell sample from a subject with a cytolytic T cell specific for said complexes, and

(ii) determining lysis of at least part of said abnormal cell sample as an indication of a candidate for said treatment.

2. The method of claim 1, wherein said MHC molecule is HLA-A2.

3. The method of claim 1, wherein said MHC molecule is HLA-B44.

4. Method for treating a subject with a cellular abnormality, comprising administering to said subject an amount of an agent which provokes a cytolytic T cell response to cells presenting complexes of an MHC molecule and a tyrosinase derived peptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 7, and SEQ ID NO: 8 on their surfaces sufficient to provoke a response to abnormal cells presenting said complexes on their surfaces.

5. The method of claim 3, wherein said MHC molecule is HLA-A2.

6. The method of claim 4, wherein said MHC molecule is HLA-B44.

7. The method of claim 4, wherein said agent comprises a vector which codes for human tyrosinase.

8. The method of claim 7, wherein said agent further comprises a second vector which codes for HLA-A2.

9. The method of claim 7, wherein said agent further comprises a second vector which codes for HLA-B44.

10. The method of claim 7, wherein said vector also codes for HLA-A2.

11. The method of claim 7, wherein said vector also codes for HLA-B44.

12. The method of claim 4, wherein said agent is a sample of non-proliferative cells which present said complexes on their surfaces.

13. Method for treating a cellular abnormality comprising administering to a subject with a cellular abnormality characterized by presentation of complexes of an MHC molecule and a tyrosinase derived peptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 7 and SEQ ID NO: 8 on surfaces of abnormal cells an amount of cytolytic T cells specific for said complexes sufficient to lyse said abnormal cells.

14. The method of claim 13, wherein said MHC molecule is HLA-A2.

15. The method of claim 13, wherein said MHC molecule is HLA-B44.

16. The method of claim 13, wherein said cytolytic T cells are autologous.

17. Isolated cytolytic T cell specific for a complex of an MHC molecule selected from the group consisting of HLA-A2 and a tyrosinase derived peptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 7, and SEQ ID NO: 8.

18. The isolated cytolytic T cell of claim 17, specific for a complex of HLA-A2 and SEQ ID NO: 4.

19. The isolated cytolytic T cell of claim 17, specific for a complex of HLA-B44 and SEQ ID NO: 7.

20. The isolated cytolytic T cell of claim 17, specific for a complex of HLA-B44 and SEQ ID NO: 8.

21. Method for identifying an abnormal cell which presents a complex of an MHC molecule and a tyrosinase derived peptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 7, and SEQ ID NO: 8 on its surface comprising contacting a sample of abnormal cells with a cytolytic T cell specific for said complex and determining lysis of said abnormal cells as a determination of cells which present said complex.

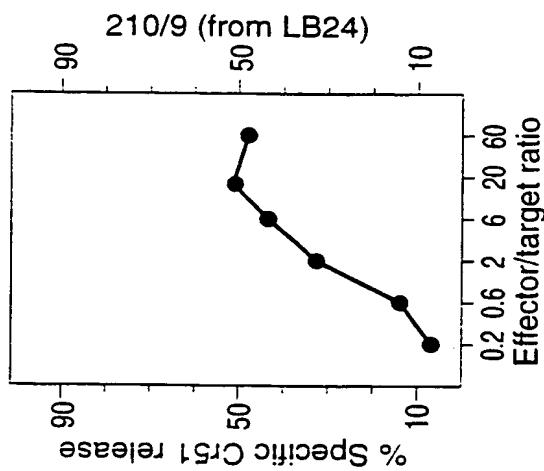
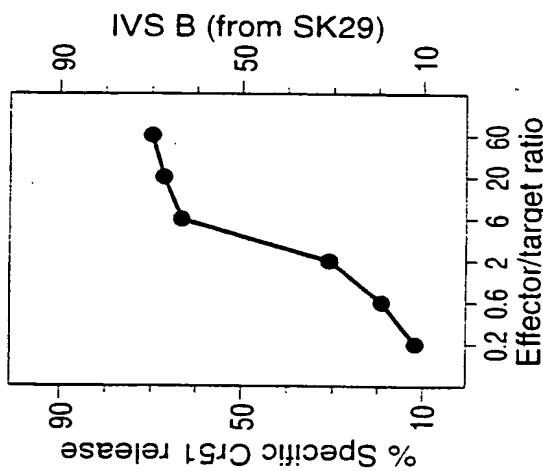
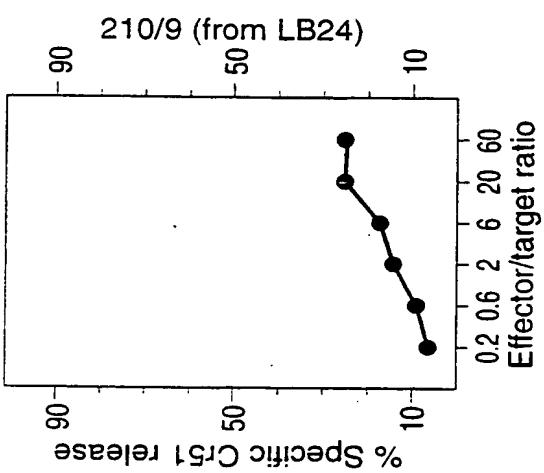
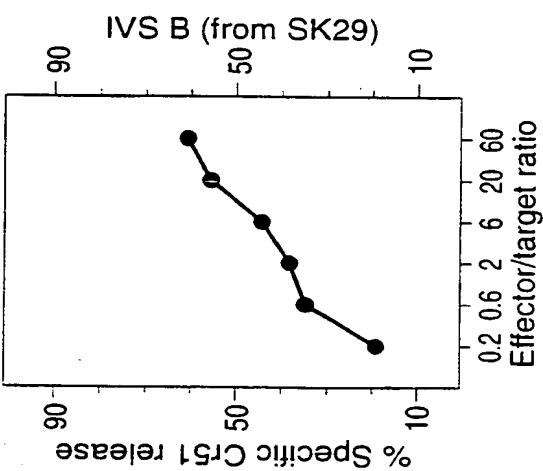
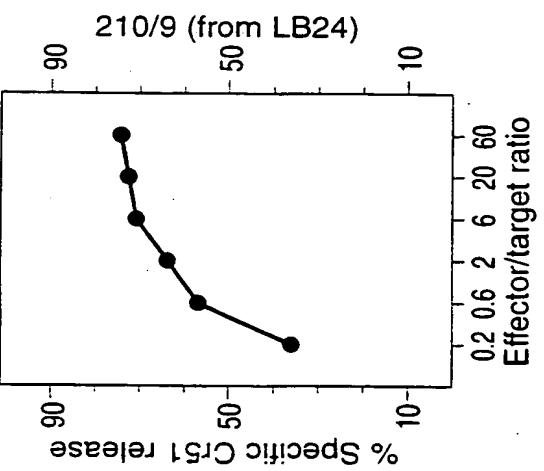
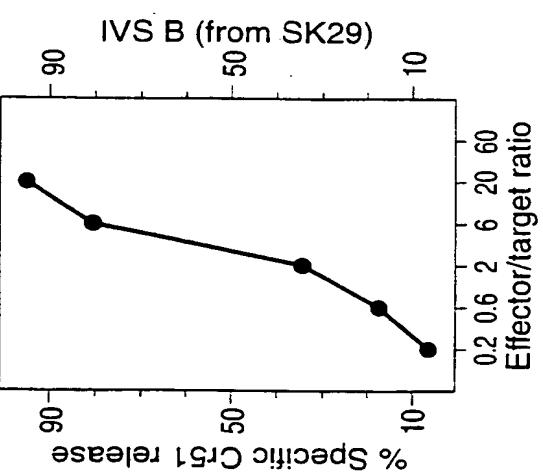
22. The method of claim 21, wherein said MHC molecule is HLA-A2.

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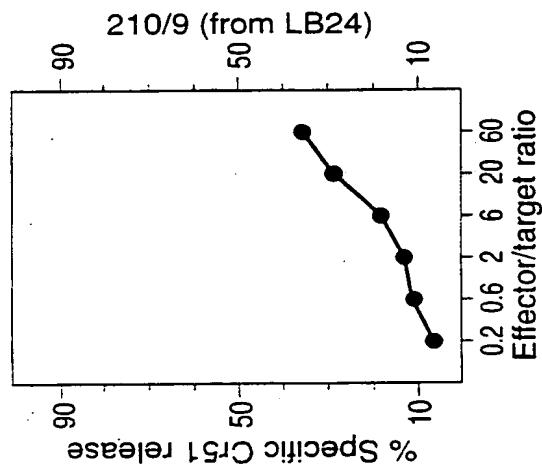
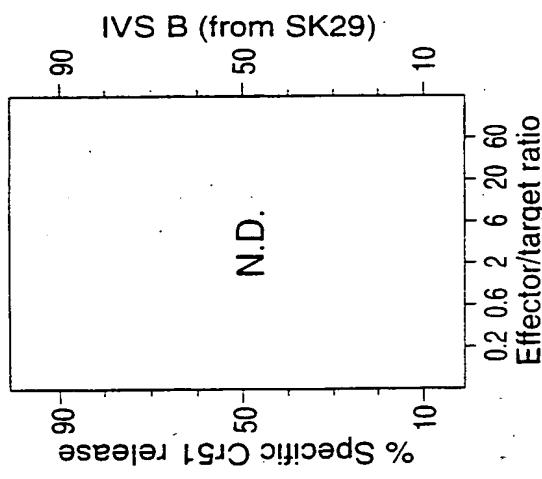
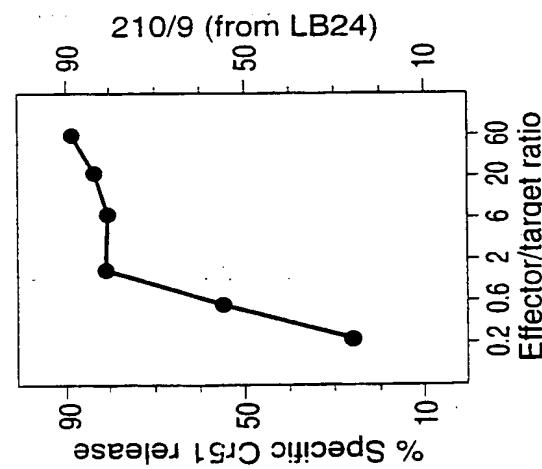
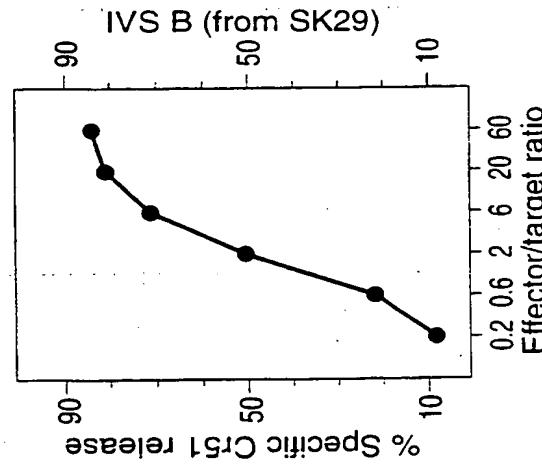
23. The method of claim 21, wherein said MHC molecule is HLA-B44.

24. Isolated peptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 7, and SEQ ID NO: 8.

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FIG. 1C**FIG. 1C'****FIG. 1B****FIG. 1B'****FIG. 1A****FIG. 1A'**

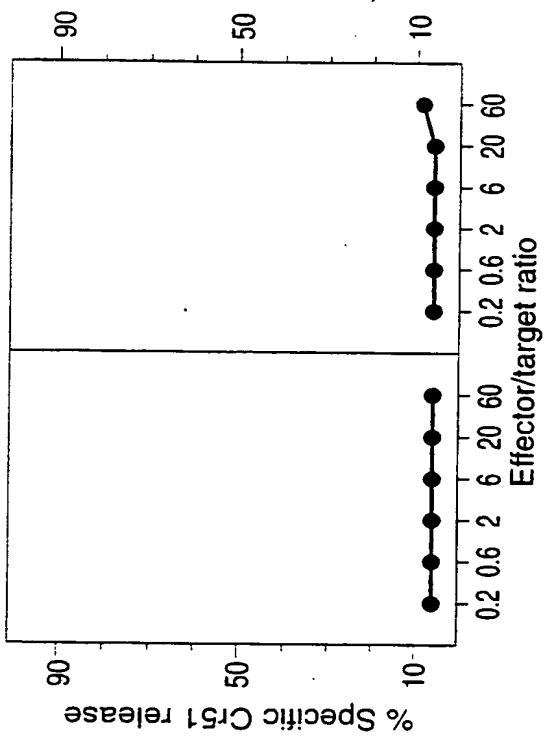
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FIG. 1E**FIG. 1E'****FIG. 1D****FIG. 1D'**

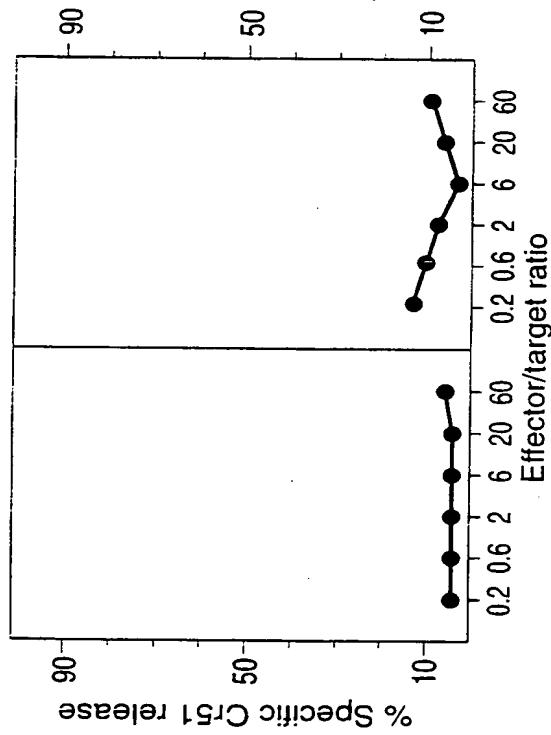
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FIG. 1F'

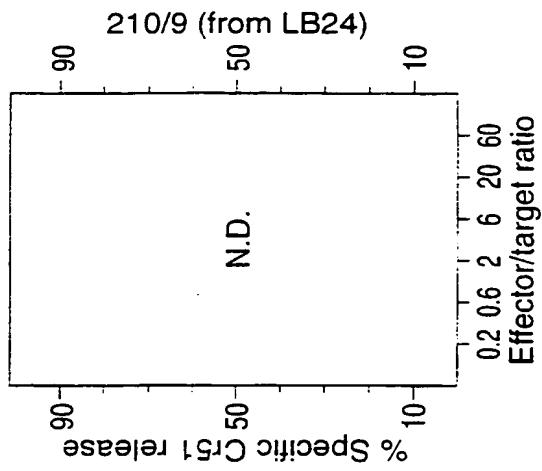
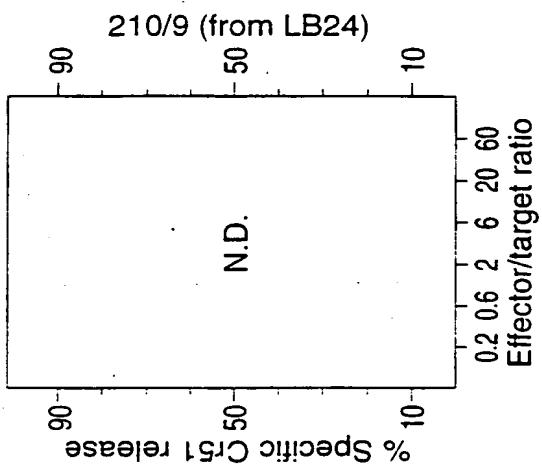
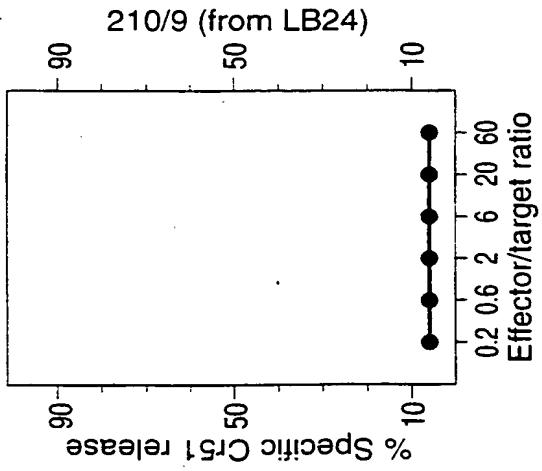
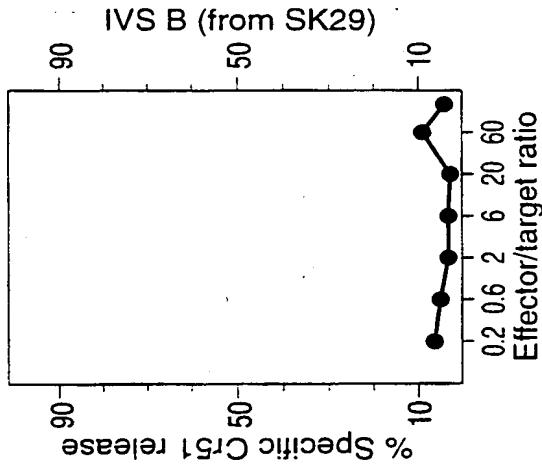
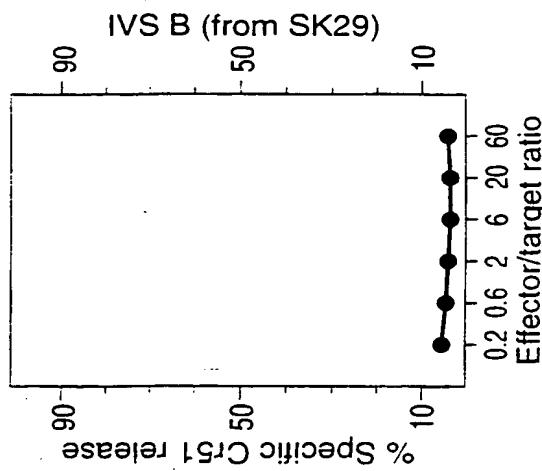
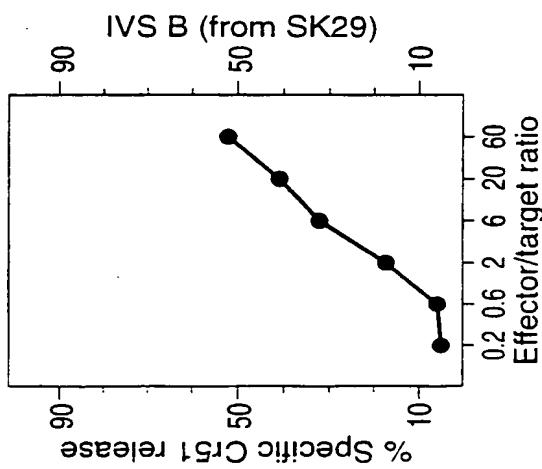
210/9 (from LB24)



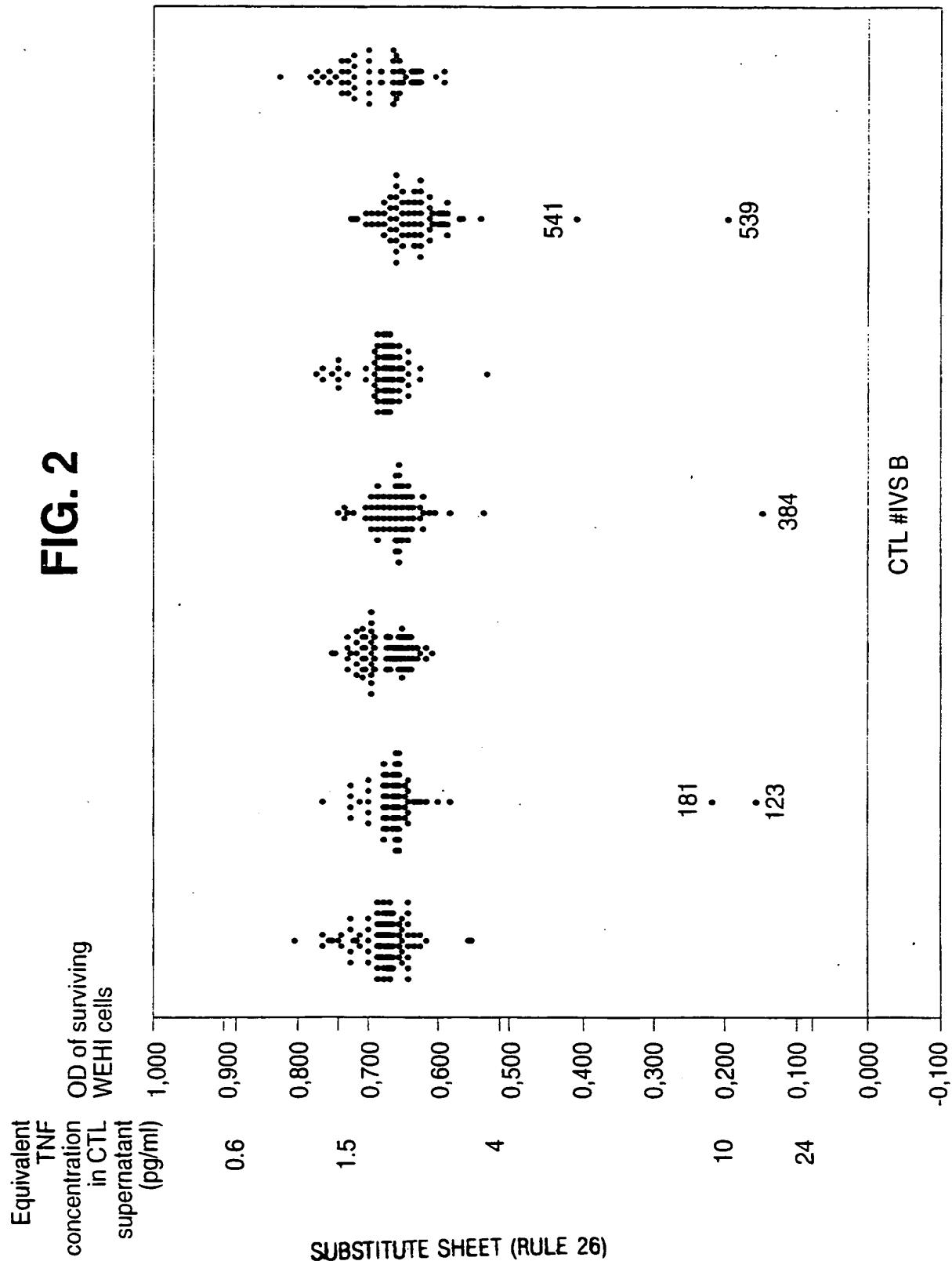
IVS B (from SK29)

**FIG. 1F**

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FIG. 11**FIG. 1H****FIG. 1G****FIG. 1G'****FIG. 1I'****FIG. 1H'**

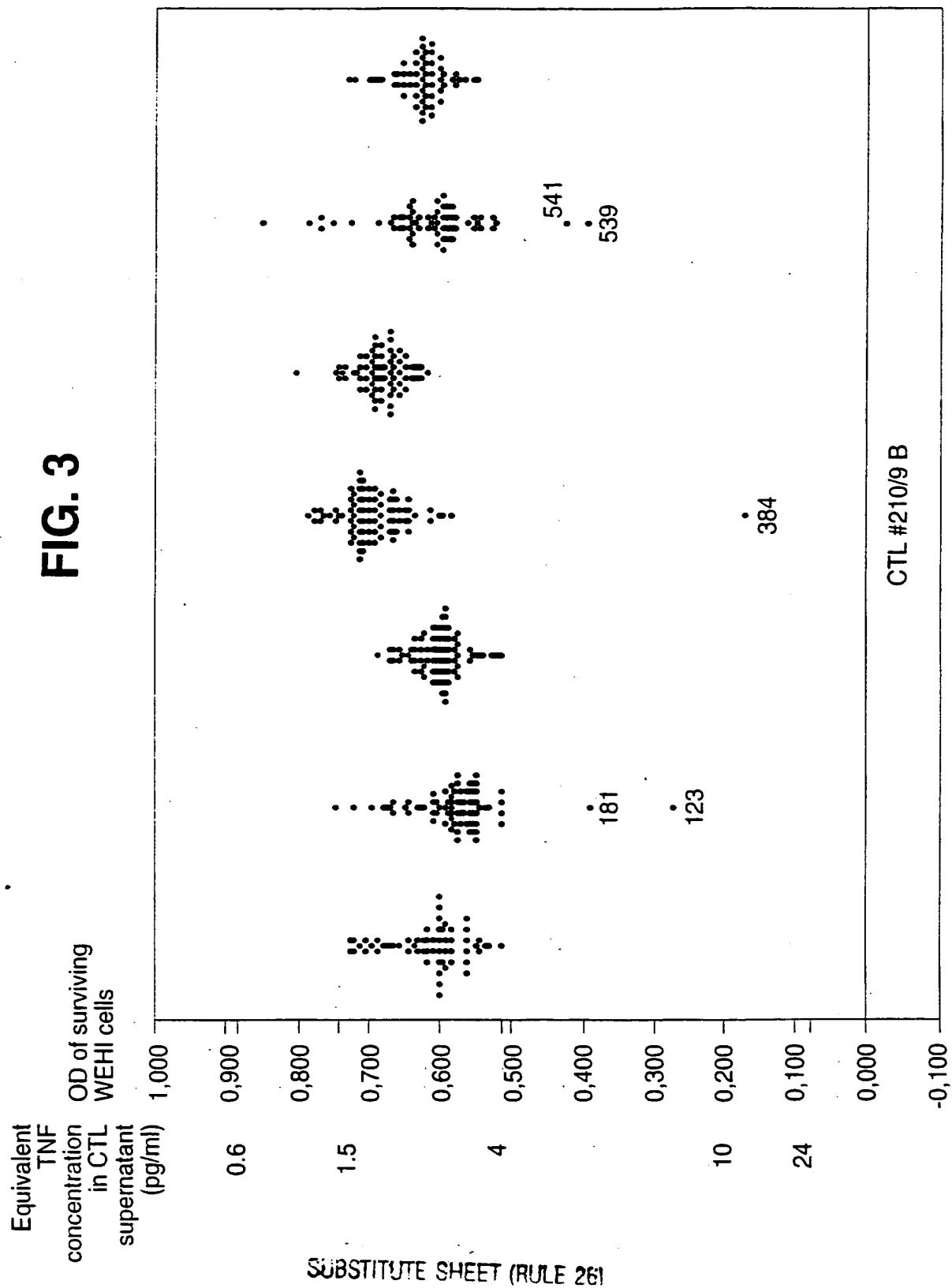
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FIG. 2

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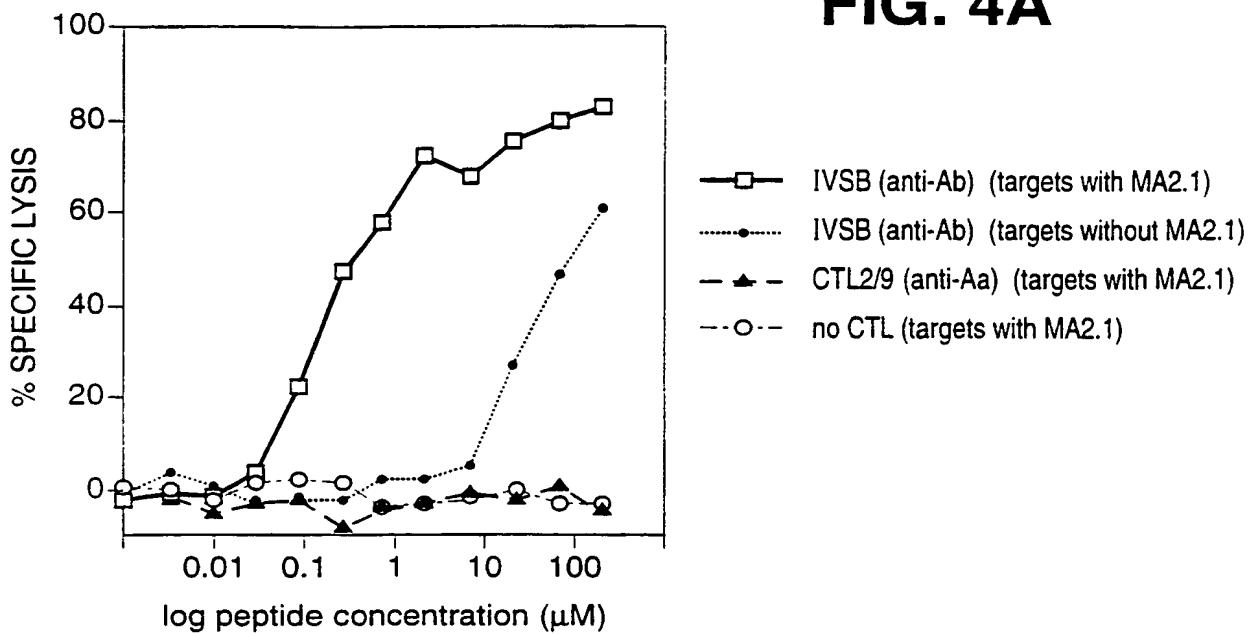
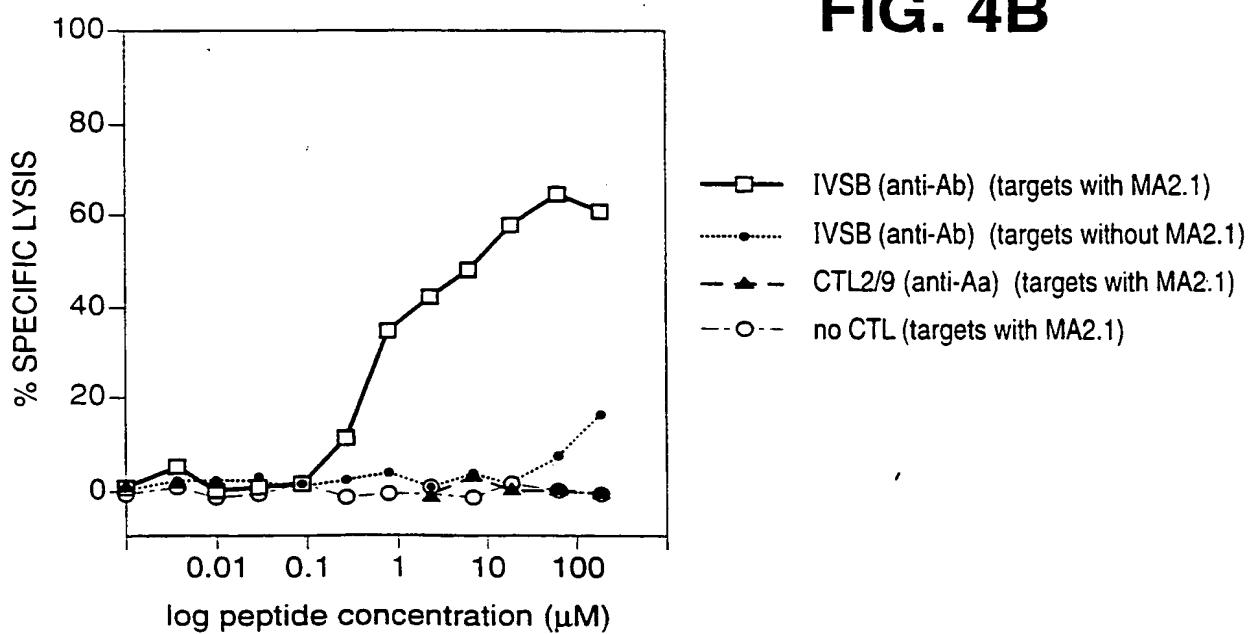
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3
FIG



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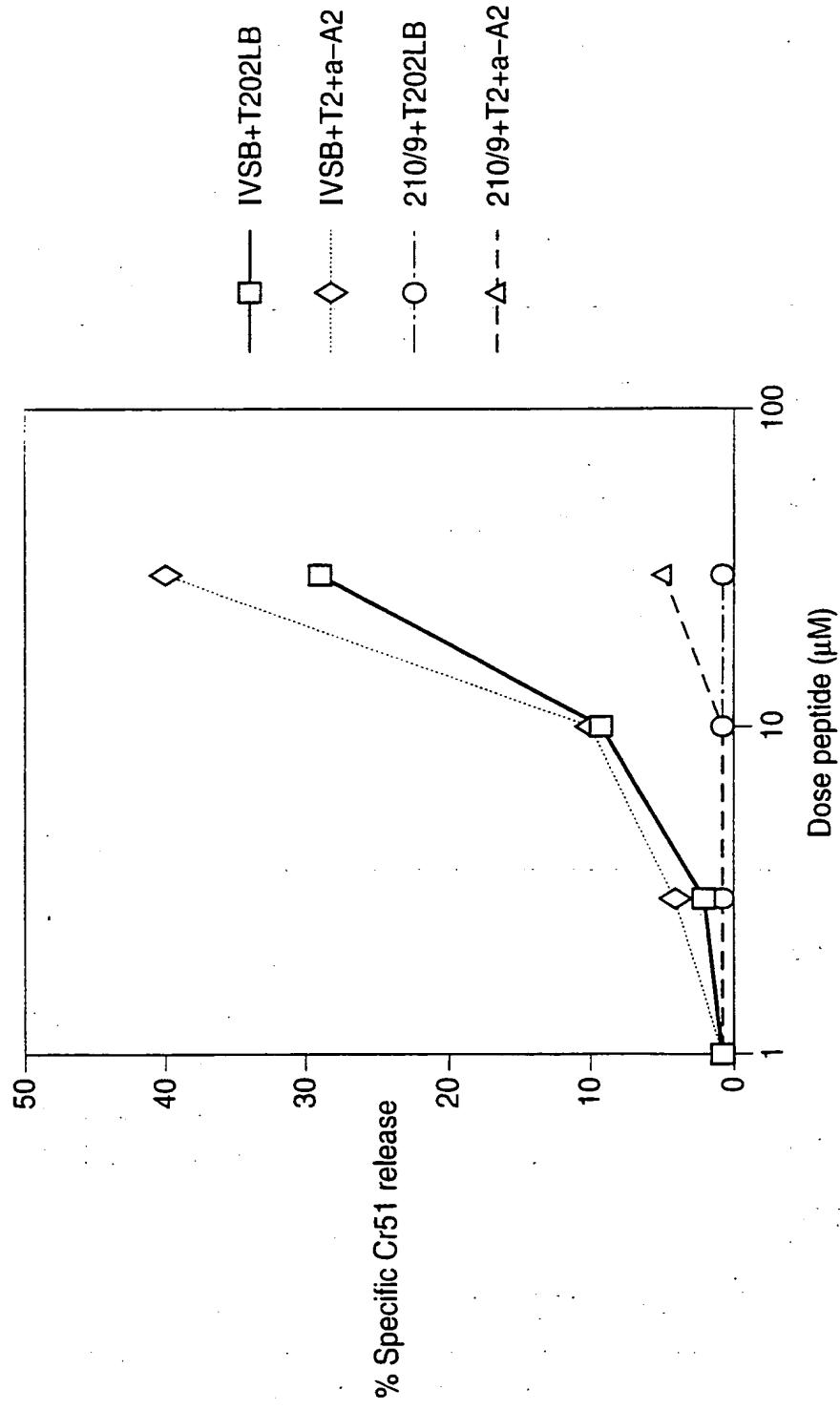
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FIG. 4A**FIG. 4B**

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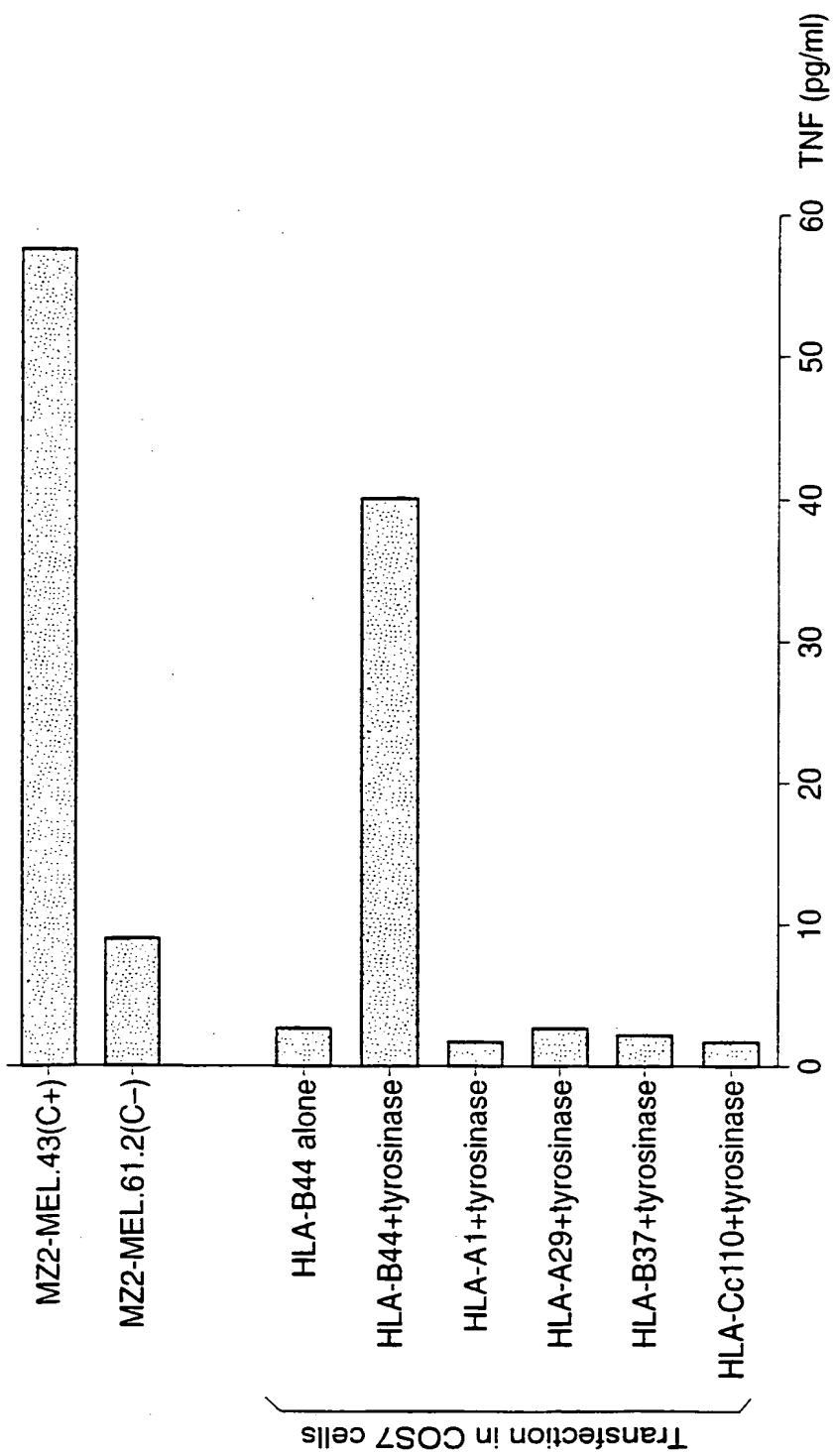
FIG. 5



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FIG. 6



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FIG. 7C

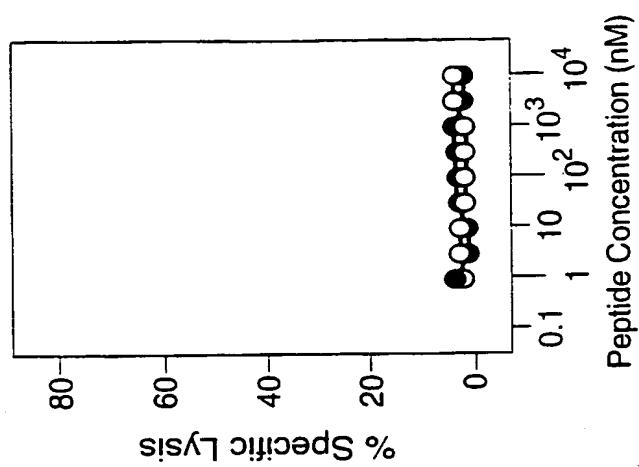


FIG. 7B

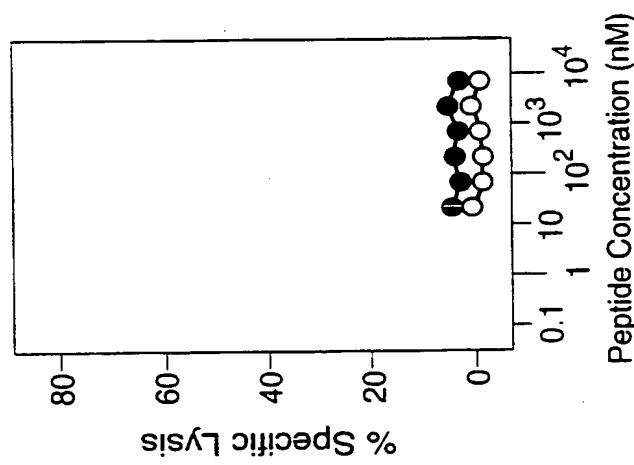
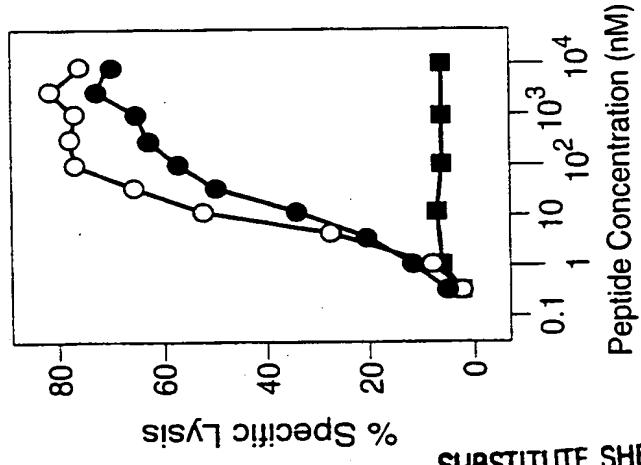


FIG. 7A



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FIG. 7D

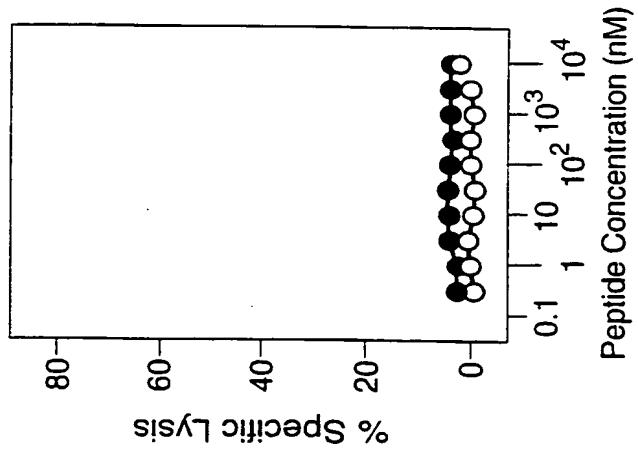


FIG. 7E

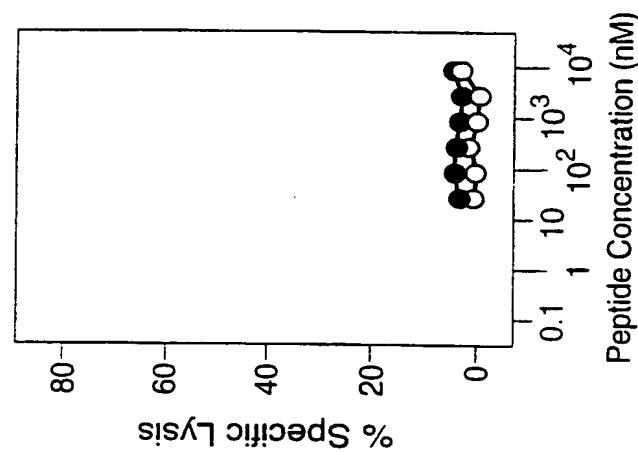
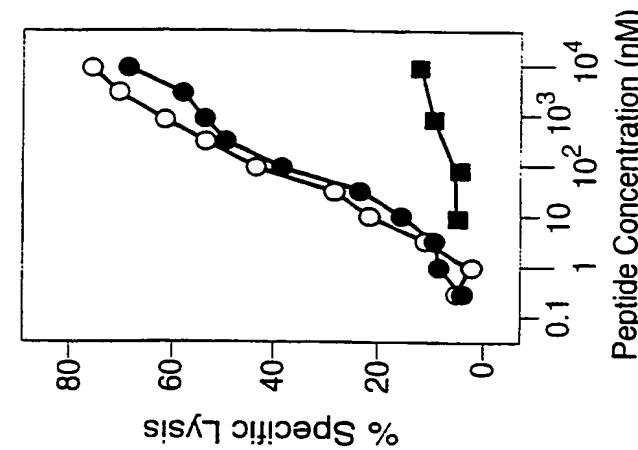
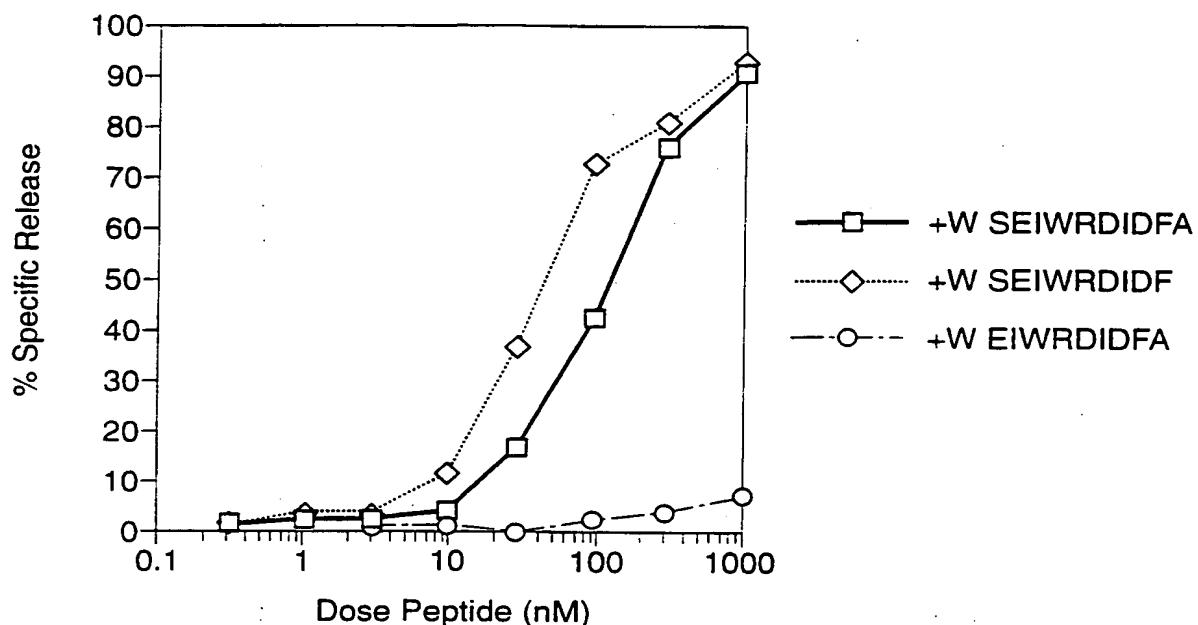
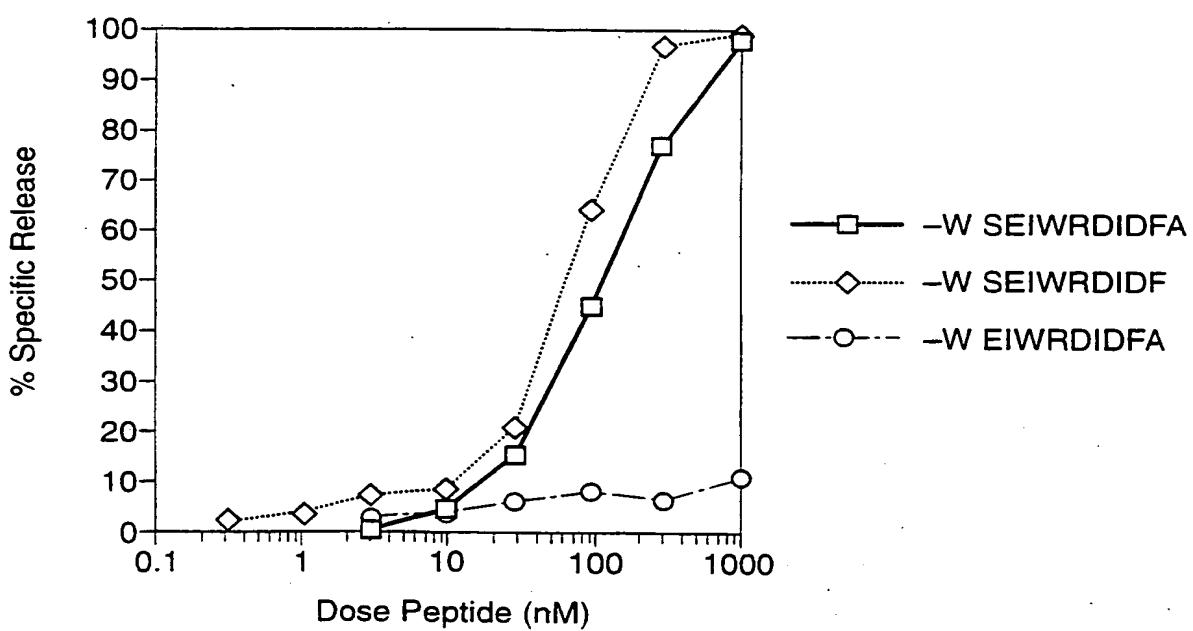


FIG. 7F



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FIG. 8A**FIG. 8B**

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01990

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/04; C12N 5/08; C07K 7/06
US CL :435/7.24, 240.2; 530/328

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/ 93.21; 435/7.23, 7.24, 240.2; 514/ 15, 16; 530/328

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, SEQUENCE SEARCH (SEQ ID NOs: 4, 7, 8)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P -----	EUROPEAN JOURNAL OF IMMUNOLOGY, Vol. 24, issued March 1994, Wolfel et al., "Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes," pages 759-764, see page 761, Figure 3.	24 -----
Y, P	I. ROITT et al., "Immunology, 3rd Edition," published 1993 by Mosby (St. Louis, Mo, USA), pages 6-10-6.11, see paragraph bridging pages 6.10-6.11.	1-3, 17-23
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 178, issued August 1993, Brichard et al., "The Tyrosinase Gene Codes for an Antigen Recognized by Autologous Cytolytic T Lymphocytes on HLA-A2 Melanomas," pages 489-495, see entire document.	1-3, 17-18, 21-24
		1-3, 17-24

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
• "A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
• "E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
• "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
• "O" document referring to an oral disclosure, use, exhibition or other means		
• "P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

08 JUNE 1995

Date of mailing of the international search report

19 JUN 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ROBERT D. BUDENS

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/01990

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INTERNATIONAL JOURNAL OF CANCER, Vol. 55, issued 1993, Wolfel et al., "Analysis of Antigens Recognized On Human Melanoma Cells By A2-Restricted Cytolytic T Lymphocytes (CTL)," pages 237-244, see entire document.	1-3, 17-24
Y	JOURNAL OF IMMUNOTHERAPY, Vol. 14, issued 1993, Coulie et al., "Genes Coding For Tumor Antigens Recognized By Human Cytolytic T Lymphocytes," pages 104-109, see entire document.	1-3, 17-24
Y,P	CANCER RESEARCH, Vol. 54, issued 15 June 1994, Robbins et al., "Recognition of Tyrosinase By Tumor-Infiltrating Lymphocytes from a Patient Responding to Immunotherapy," pages 3124-3126, see entire document.	1-3, 17-24
A	THE JOURNAL OF IMMUNOLOGY, Vol. 150, No. 7, issued 01 April 1993, Slingluff, Jr. et al., "Recognition of Human Melanoma Cells by HLA-A2.1-Restricted Cytotoxic T Lymphocytes Is Mediated by at Least Six Shared Peptide Epitopes," pages 2955-2963.	1-3, 17-24
T	THE JOURNAL OF IMMUNOLOGY, Vol. 154, issued 1995, Visseren et al., "CTL Specific for the Tyrosinase Autoantigen Can Be Induced from Healthy Donor Blood to Lyse Melanoma Cells," pages 3991-3998, see entire document.	1-3, 17-24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/01990

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-3, 17-24
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01990

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-2, 17-18, 21-22 and 24, drawn to methods of identifying candidates for therapy.

Group II, claims 4-12, drawn to a second method, methods of treatment using a therapeutic agent.

Group III, claims 13-16, drawn to a third method, methods of treatment using cytotoxic T lymphocytes.

Group IV, claim 24, drawn to isolated tyrosinase peptides.

The inventions listed as Groups I-III and IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions of Groups I-III are directed to distinct methods using different method steps and uses. Further, the product of Group IV is independent of the methods encompassed in the inventions of Groups I-III. The product of Group IV does not share a special technical feature with the methods of Groups I-III.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Group V, claims 3, 19-20 and 23, drawn to a second HLA haplotype, HLA-B44.

Group VI, claims 1-3 and 17-24, drawn to a second species of tyrosinase peptide, SEQ ID NO: 7.

Group VII, claims 1-3 and 17-24, drawn to a third species of tyrosinase peptide, SEQ ID NO: 8.

The following claims are generic: 1, 21

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Group V is directed to a different HLA haplotype distinct from HLA-A2 and differing in structure and function from HLA-A2. Further, the species of Groups VI-VII are directed to tyrosinase peptides differing in their primary amino acid sequence, structure and physical properties and are distinct from the tyrosinase peptide of SEQ ID NO:4. Groups V-VII do not share a special technical feature.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

